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ROLE OF WNT SIGNALLING IN FOREBRAIN DEVELOPMENT

DAVID ANTHONY MCLAUGHLIN

Thesis submitted for the degree of Doctor of Philosophy in the Faculty of Medicine,

University of Edinburgh.

March 2002

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Disclaimer

I certify that I (David McLaughlin) performed all of the experiments presented in this thesis unless otherwise clearly stated below. No part of this work has been, or is being submitted for any other degree or qualification.

1. In Chapter three, *in situ* hybridisation for Wnt8B at E9.5 in Figure 1. was carried out by Dr. John Mason.
2. In Chapter five, Golgi cox staining and cytochrome oxidase staining were performed a project student, Fredrick Karlesson under my supervision.

   Photomicrographs in Figure 5.1 were from work carried out by Dr V. Wilson

Signed:

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Abbreviations

ANR :Anterior neural ridge
AHPs :Adult hippocampal progenitors
AP :Anterior-posterior
BrdU :5-bromo-2-deoxyuridine
CNS :Central nervous system
CP :Cortical plate
CPE :Choroid plexus epithelium
Ctx :Cortex
DAB :3,3'-diaminobenzidine
ddH_20 :Double distilled water
DG :Dentate Gyrus
DNA :Deoxyribonucleic acid
DV :Dorsal-ventral
E :Embryonic day
FP :Floor plate
Fz :Frizzled
GABA :γ-aminobutyric acid
GAG :Glycosaminoglycans
GL :Granular layer
H :Hylus
H&E :Haematoxylin and eosin
HS :Heparan sulphate
HSPG :Heparan sulphate proteoglycan
ISH :*In situ* hybridisation
I :Isthmus
IC :Internal capsule
IZ :Intermediate zone
LTW :Lateral telencephalic wall
LV :Lateral ventricle
MF :Mossy fibre
mg :Milligram
mRNA :Messenger ribonucleic acid
MTW :Medial telencephalic wall
MZ :Marginal zone
P :Postnatal day
PBS :Phosphate buffered saline
PG :Proteoglycans
PCP :Planar cell polarity pathway
Pcp :Precaudal plate
PCR :Polymerase chain reaction
PKC :Protein kinase C
PP :Perforant pathway

PPL :Primordial plexiform layer
PZ :Proliferative zone
RMS :Rostral migratory stream
RP :Roof plate
RT :Room temperature
SCH :Shaffer collateral pathway
SFRP :Secreted frizzled related proteins
SGL :Sub-granular layer
SP :Subplate
SPP :Secondary proliferative population
SVZ :Subventricular zone
TBS :Tris buffered saline
TCT :Thalamo-cortical tract
µg :Microgram
Wg :Wingless
Zli :Zona limitans intrathalamica

Abstract

The mammalian cerebral cortex is probably the most complex and highly organised structure to have evolved; yet the major part of its development is completed in a relatively short period from its emergence from the anterior neural tube at around ten days of embryonic development in the mouse. The processes driving this development are under the control of the products of regulatory genes.

The application of modern techniques of molecular biology has led to the understanding of the effects of many of these genes, though the contribution of many other genes remains to be discovered. This study investigates the role of two genes involved in the Wnt signalling pathway, which plays a crucial role in embryogenesis.

In situ hybridisation (ISH) was used to determine the expression pattern of a new member of the Wnt gene family *Wnt8B*. During early development *Wnt8B* expression was observed in the cerebral cortical hem region which is presumptive for the hippocampus. In the adult, *Wnt8B* is expressed in the dentate gyrus region of the hippocampus, one of the areas in which adult neurogenesis takes place. To investigate the role of *Wnt8B* in regulating central nervous system development we undertook histological analysis and 5-bromo-2-deoxyuridine (BrdU) studies in $Wnt8B^{+/+}$ and $Wnt8B^{-/-}$ mice in regions where *Wnt8B* is expressed. Surprisingly, histological analysis showed no obvious abnormalities in $Wnt8B^{-/-}$ animals. Further, no significant differences in cell proliferation were found in BrdU studies in the $Wnt8B^{-/-}$ embryos or adult mice.

Heparan sulphate proteoglycans are structurally dynamic molecules required for Wnt signalling. The nature of specific structural modifications made to these molecules such as the sulphation pattern is thought to confer specific biological effects. To determine the role of 2-0 sulphation in cellular proliferation in the cerebral cortical hem during development, BrdU studies were undertaken in mice deficient in the enzyme heparan sulphate 2-0 sulphotransferase (Hs2st). BrdU labelling was also used to investigate cell migration in the cerebral cortex and a combination of histochemical techniques was applied to look for gross and subtle anatomical defects in the forebrain of *Hs2st*$^{-/-}$ mice. In agreement with a dramatically reduced thickness of the cerebral cortex seen in histological sections, BrdU labelling indicated proliferative rates 40% lower in *Hs2st*$^{-/-}$ mice.

CHAPTER 1: General introduction

Despite the efforts of many scientists we are still far from a complete understanding of the processes underlying the development of the nervous system, though nature replicates this complex structure with amazing regularity. To achieve this, stringent regulatory mechanisms must exist both intracellularly and in the extracellular environment providing instructions to cells, be it to divide, migrate, differentiate, interconnect with those around or far from it, or to die. Once these processes are more fully understood, we will be able to manipulate them with greater precision, potentially leading to therapeutic strategies for inherent genetic defects, as well as disorders caused by environmental injury. An understanding of the role of genes important in development, as well as the developmental process at a cellular level will be fundamental to the realisation of many of these therapies.

1.1 Neurulation

After late gastrulation in the mouse, at approximately E7.5 the dynamic process of neurulation begins. Initially, a flat neural plate is formed, extending anteriorly from the node, with an anterior-posterior groove and distinct neural ridges. As the process continues, these ridges bend over towards one another and eventually fuse at the dorsal midline to form what is termed the neural tube (Figure 1.1). The neural tube gives rise to the entire central nervous system (CNS), except for the microglia. Closure of the neural tube initiates approximately half way along the anterior-posterior axis of the neural tube

and then proceeds bilaterally, such that closure of the posterior neural tube is completed by approximately E9.5, and closure of the anterior neural tube by approximately E10.

Concurrently, differential rates of cell division at the anterior end of the neural tube produce three swellings. The anterior-most swelling forms the presumptive forebrain or prosencephalon, followed by the midbrain or mesencephalon, and the hindbrain or rhombencephalon which runs continuous with the caudal part of the neural tube.

1.2 Development of the dorsal telencephalon

The prosencephalon is further divided into the telencephalon and diencephalon. The telencephalon emerges from swellings of the lateral walls of the prosencephalon which subsequently expand to form part of the dorso-lateral cerebral cortex. The distinct structures of the striatum, ganglionic eminence and limbic system comprise the medial-ventral cortex. The central part of the prosencephalon gives rise to the thalamus, dorsally and the hypothalamus, ventrally.

The cerebral cortex is a laminated structure, as illustrated in Figure 1.2. At approximately E12 in the mouse, the cortex is divided into an inner layer of proliferating cells termed the ventricular zone (VZ), and an outside layer known as the primordial plexiform layer (PPL) or preplate (Boulder Committee, 1970; Marin-Padilla, 1971). Subsequently, cells from the ventricular zone start to accumulate within the preplate, splitting it into the marginal zone (MZ) and a transient layer of cells called the subplate (SP)(Luskin & Shatz, 1985; Allendoerfer & Shatz, 1994). At around E14, migrating

cells from the ventricular zone separate the marginal zone and subplate to form layers six and five. Further waves of migrating cells then form layers four to two respectively, layer one being formed by the cells of the marginal zone. Hence the cortical layers are formed in an inside first, outside last manner.

Neurogenesis proceeds in this way until around E17 when there is a transition to predominantly glial cell production. From around E14, glial cells are produced from a secondary proliferative population (SPP) which is diffusely located in the subventricular and intermediate zones immediately above the ventricular zone (Altman & Bayer, 1990; Takahashi et al, 1995). Further, a specialised group of cells termed radial glia which perform a guidance role for migrating cells have additionally been shown to act as precursurs for neurons and glia (Malatesta et al, 2000; Hartfuss et al, 2001). As cortical cells reach their final destination, they complete differentiation and form appropriate connections with their target cells.

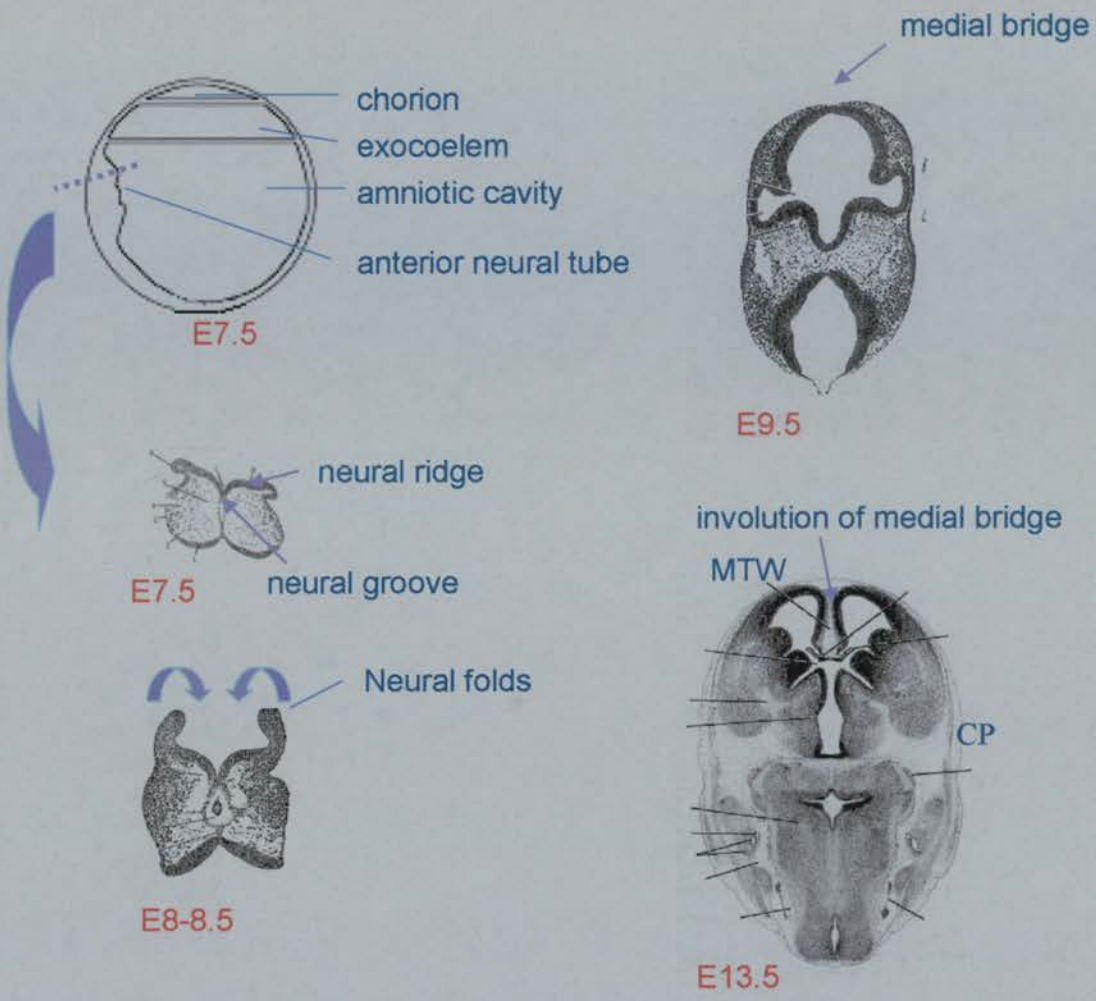


Figure 1.1 Overview diagram illustrating the closure of the anterior neural tube. A schematic cross section through the embryonic capsule at E7.5 illustrates the position of the developing prosencephalon (dotted line and arrow indicates the plane of section shown in the other pictures). A developmental sequence of photomicrographs from E7.5 show the lateral tips of the neural plate in the process of folding over and then fusing at around E9.5 to form the medial bridge. Subsequently the medial bridge involutes to form the medial wall which is prominent by E13.5. Abbreviations: MTW, medial telencephalic wall; CP, choroid plexus. Photomicrographs reproduced from M Kaufman (1992).

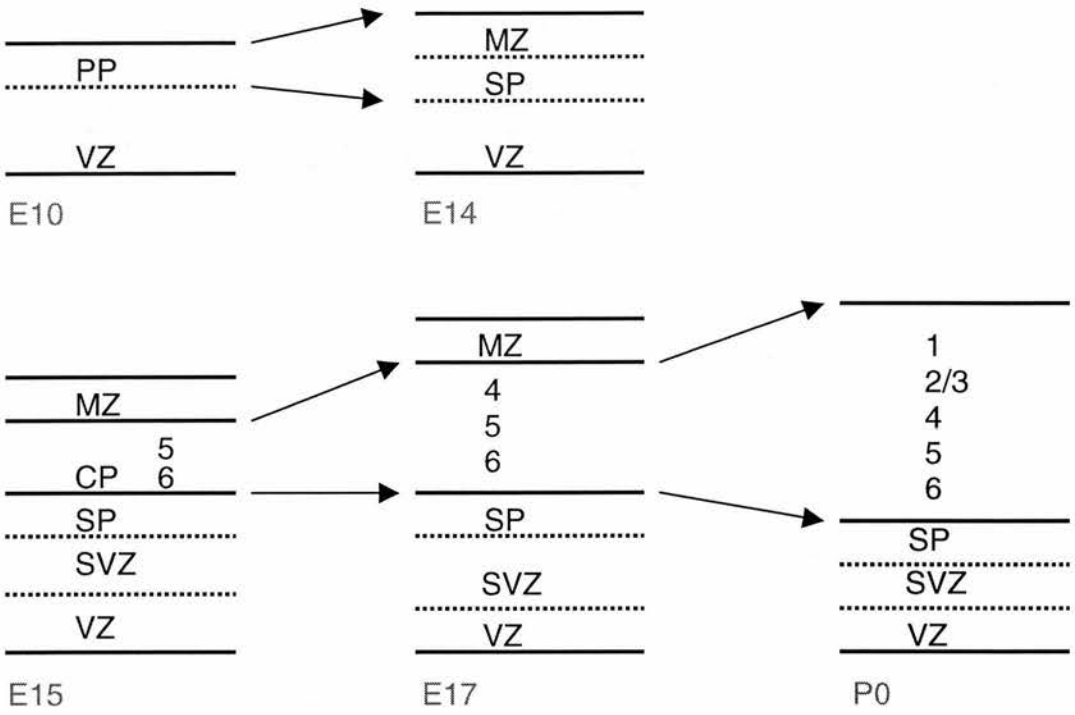
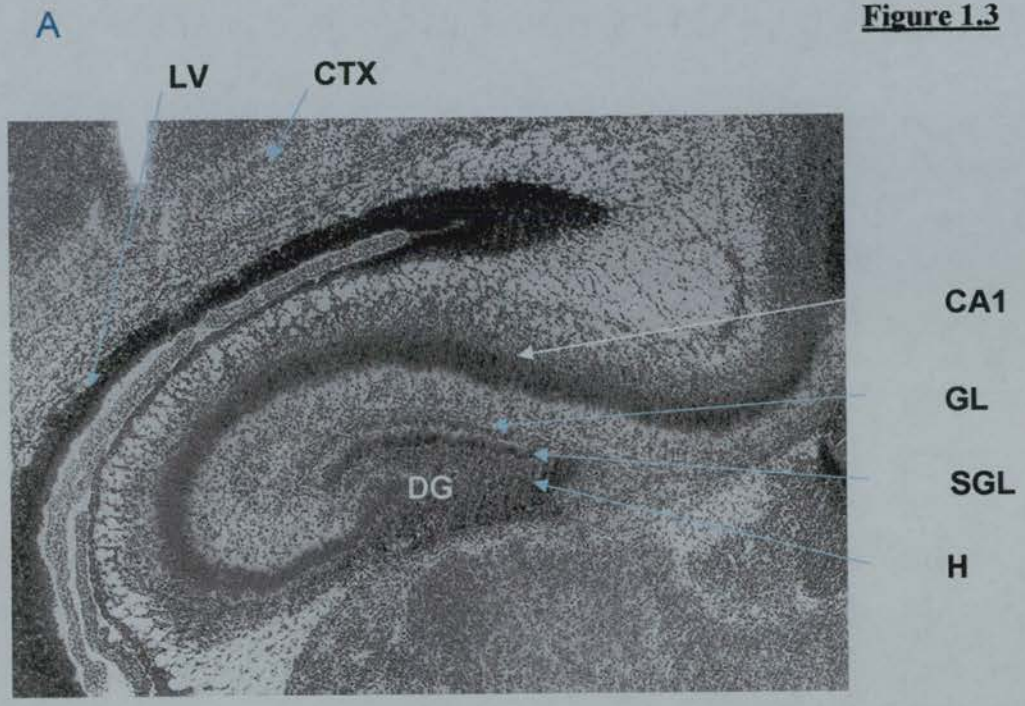


Figure 1.2 Diagram illustrating the development of cortical lamination. At E10 the neuroepithelium of the cortex is composed of a ventricular zone which is only a few cells thick. As described in the text, cells born in the ventricular zone migrate to expand the cortical plate and form specific layers which are dependent on the cells birth date. Abbreviations: PP, pre-plate; VZ, ventricular zone; MZ, marginal zone; SP, subplate; CP, cortical plate; SVZ, sub ventricular zone.

1.2.1 Development of the medial wall of the telencephalon

The medial walls of the telencephalon are formed by the invagination of the superior bridge, which begins at around E10.5 after closure of the anterior neural tube (Figure 1.1). At E12.5 this narrow strip of epithelium starts to develop into a number of distinct structures. The most ventral part gives rise to the choroid plexus (which is comprised of non neuronal cuboidal secretory epithelium and regulates the ventricular fluid). Two days later, stratified epithelium, which is contiguous to the choroid plexus dorsally, starts to expand and folds to form the hippocampus. The hippocampus is a specialised structure involved in the acquisition of short term memory (Squire & Zola-Morgon, 1989). The hippocampus can be divided into three regions which have been traditionally designated CA1-CA3. The dentate gyrus, subiculum and entorhinal cortex are included in the more general term hippocampal formation (Figure 1.3). Both the hippocampus and the dentate gyrus are comprised of three layers, the polymorphic layer, the pyramidal layer and the molecular layer. Sensory input is transmitted from the entorhinal cortex by the perforant pathway which is topographically organised into two distinct projections which contribute fibres to all hippocampal fields (Amaral 1993)(See Figure 1.3b). Mossy fibre projections from the granule cells of the dentate gyrus terminate on the pyramidal cells of CA3 which in turn project to CA1. Connections also occur from CA1 to CA3. The outflow of information from the hippocampus occurs via the subiculum to cortical association areas, and via commissural connections to the contralateral hippocampus (Frotscer & Heimrich 1993).

Figure 1.3


A
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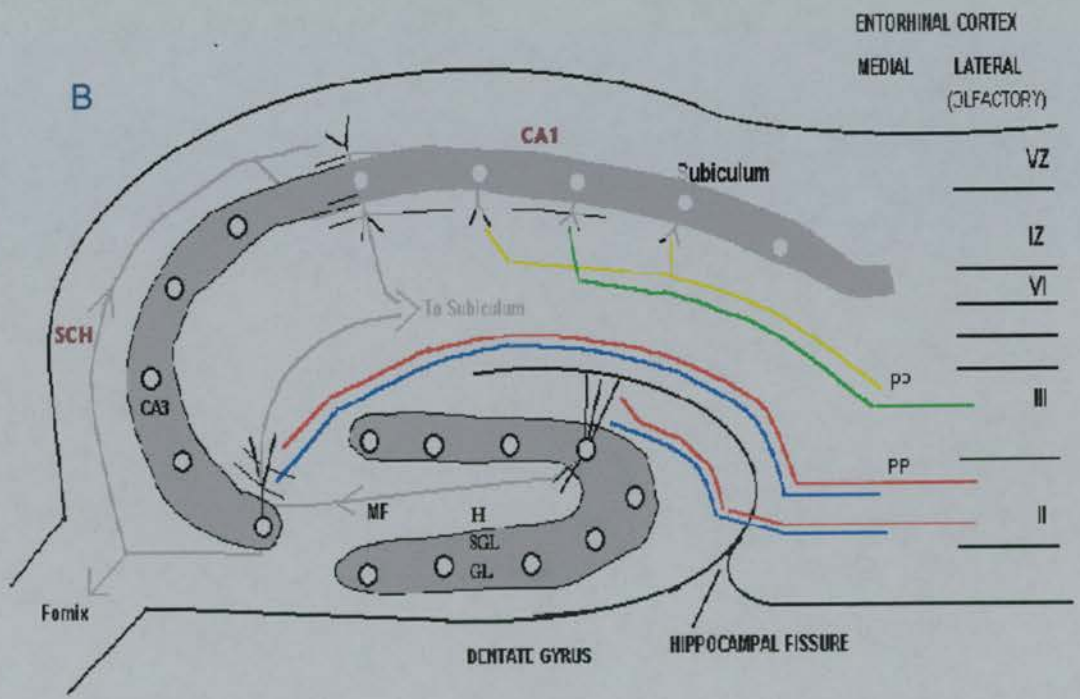
B
ENTORHINAL CORTEX
MEDIAL
LATERAL
(OLFACTORY)
CA1
Subiculum
VZ
IZ
VI
To Subiculum
SCH
CA3
PP
III
PP
MF
H
SGL
GL
II
Fornix
DENTATE GYRUS
HIPPOCAMPAL FISSURE

Figure 1.3 Illustration of hippocampal anatomy and circuitry. (A) coronal section through the adult hippocampus shows the cell layers of the hippocampus and dentate gyrus. The large pyramidal cells of CA1-CA3 form a continuous layer which is interlocking with the dentate gyrus, the sub granular layer of which generates new neurons during adulthood. This structure is continuous with the cortex dorsally but is separated from it laterally by the walls of the lateral ventricle, the second site of adult neurogenesis in the adult brain. (B) Neurons of the entorhinal cortex project to the hippocampus and dentate gyrus in a highly organised manner. Two perforant pathways project onto the dentate gyrus and all regions of the hippocampus. One originates from cortical layer three (distinquished in the digram as green/yellow), and the second originates from cortical layer two (red/blue). A topographical projection is maintained from the medial and lateral cortex within each pathway, to distinct regions of the dentate gyrus and hippocampus, (this division within both pathways is illustrated in the diagram by green and yellow, and by red and blue). This topography is maintained at points along the CA fields and subiculum. Hippocampal neurons project to adjacent cortical association areas, to the hypothalamus via the fornix, and to the contralateral hippocampus. Abbreviations: DG, dentate gyrus; H, hilus; SGL, sub granular layer; GL, granular layer; PP, perforant pathway; MF, mossy fibre pathway; SCH, schaffer commisural pathway; LV, lateral ventricle; CTX, cerebral cortex.

A separate, distinct commissural pathway is formed by the corpus callosum. This is the second largest efferent axonal pathway from the neocortex and crosses the dorsal midline of the medial telencephalic wall at the level of the choroid plexus. Axons from the presumptive cingulate cortex which are generated at E14, first cross the midline at E17 (Koester & O'Leary D, 1994). The most dorsal part of the medial wall of the telencephalon forms the dorso-medial most aspect of the neocortex which develops as described above.

On the basis of *Wnt* gene expression at the medial edge of the telencephalon, the boundary between the hippocampus and choroid plexus has been called the cortical hem (Grove et al. 1998). By birth, much of the growth and patterning of choroid plexus epithelium and hippocampus is complete, and the cortical hem, as defined by multiple *Wnt* gene expression, disappears (Tole et al, 1997).

The genetic control of forebrain development has been difficult to study due to the lack of histological segmentation and because relatively few genes have been described which are selectively expressed there, and potentially involved in morphogenesis. A member of the *Gli* family of transcription factors ,*Gli3*, has been found to be selectively expressed in the developing anterior-dorsal neural tube. A mouse containing a deletion mutation in this gene (termed *extra-toes*, *Xt*), does not develop midline structures and has a malformed cerebral cortex which has no lamination (Franz, 1994). *Gli3* is a vertebrate homologue of the Drosophila gene *cubitus interuptus*, which encodes a transcriptional regulator of the Drosophila *Wnt* gene, *Wingless* (Wallis & Muenke,

1999). In the extra-toes mutant, expression of *Wnt2B, Wnt3A* and *Wnt5A* were found to be downregulated specifically in the dorso-ventral wall of the telencephalon, suggesting the involvement of *Gli3* in normal development of the medial telencephalic wall, and in the regulation of *Wnt* gene expression (Grove et al, 1998).

1.2.2 Cell proliferation

In order to divide, progenitor cells in the ventricular zone go through a sequence of four phases termed the cell cycle, during which their nuclei move intracellularly (Figure 1.4). In S phase, the nucleus is positioned at the outside edge of the ventricular zone. DNA replication occurs during this phase. During the next phase termed G2, the nucleus moves to the ventricular surface and the cell then undergoes mitosis (M phase). After M phase, the daughter cells either re-enter the cell cycle or become postmitotic and migrate within the cortical plate.

Many studies have been carried out to examine the cell cycle in the developing cortex. These have mainly involved labelling of newly synthesised DNA using tritiated thymidine ([^{3}H]Th) or 5-bromo-2-deoxyuridine (BrdU) (Takahashi et al, 1992; Waechter & Jaensch, 1972). These studies have revealed an increase in the length of the cell cycle from approximately 8.1 hours at E11 to 18.4 hours at E18 (Angevine & Sidman 1961; Luskin & Shatz 1985; Rakic 1974).

Recent evidence supports the theory that two types of cell division occur in the ventricular zone, symmetric and asymmetric cell division (Chenn & McConnell 1995;

Mione et al 1997). During symmetric division the cell is cleaved perpendicular to the basal lamina of the ventricular zone and the result is two daughter cells which become multipotent progenitor cells, or stem cells. During asymmetric division the cell is cleaved parallel to the basal laminae. This results in the production of one progenitor cell which will remain in the ventricular zone and may re-enter the cell cycle, and one post-mitotic cell which may migrate and differentiate within the cortical plate. Symmetric division may thus be used to amplify the number of progenitor cells earlier on in development whereas asymmetric division may be important later to produce the vast numbers of post mitotic neurons needed.

In addition to the temporal gradients of neurogenesis and gliogenesis mentioned above, regional gradients also exist within the cortex. A general high to low gradient of rates of proliferation is found from the caudal to rostral, and lateral to medial directions in the cortical plate (Bayer & Altman, 1991). Subtler differences must also exist in the developing cortical epithelium, as changes in local proliferative rates must underlie the development of diverse regional anatomical features such as the choroid plexus and hippocampus.

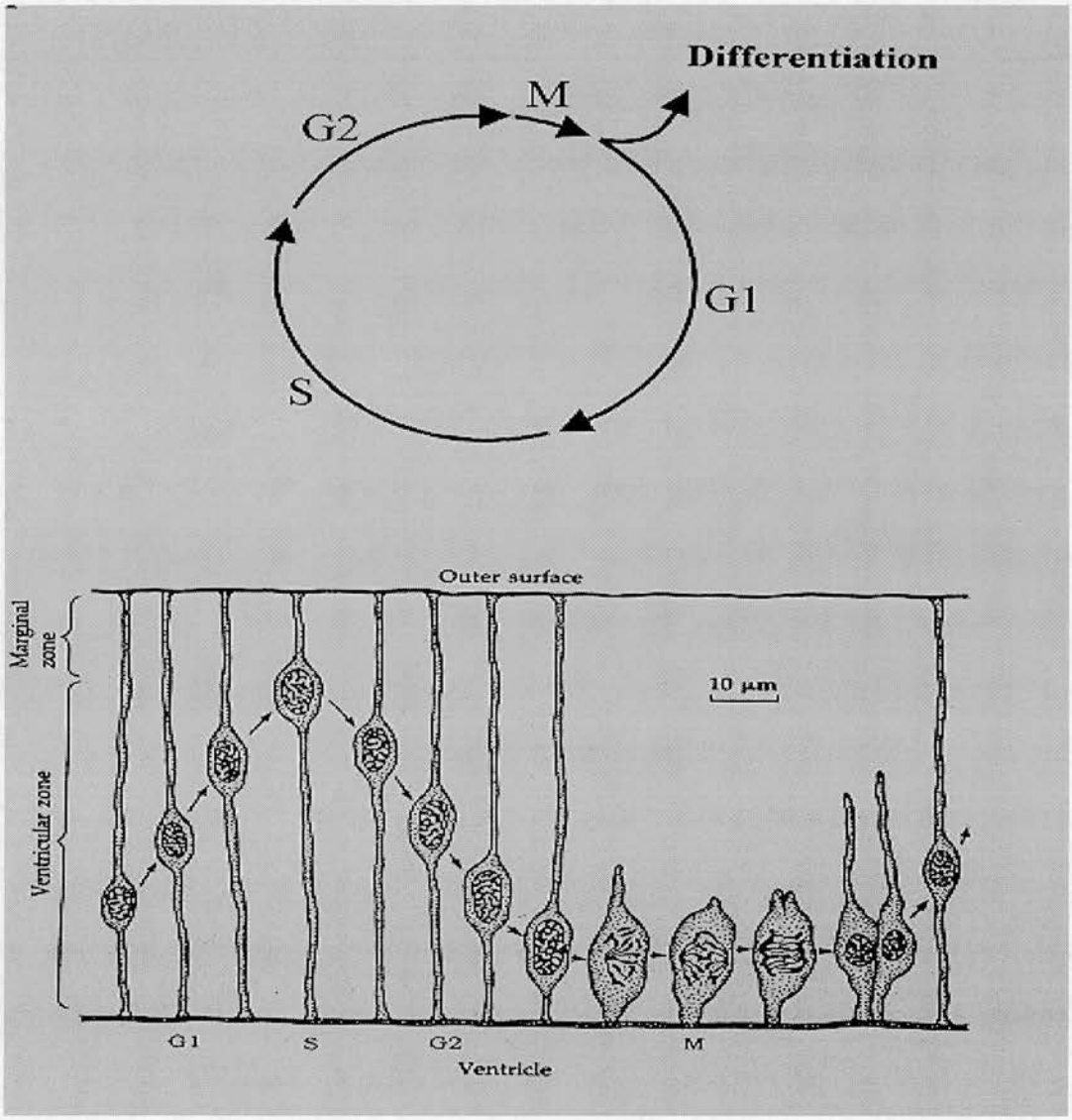

Figure 1.4 Diagram illustrating the cell cycle in cells of the cortical ventricular zone (the lengths of the cell cycle in the cortex has been determined, see 1.2.2). The cell cycle occurs in the ventricular zone epithelium. During G1, progenitor cell nuclei migrate to the outer edge of the epithelium where they undergo S phase. During G2, the nucleus moves back to the ventricular edge where mitosis (M phase) occurs. Reproduced from Purves and Lichtman (1985).

1.2.3 Cell migration

After the advent of electron microscopy and tritiated thymidine autoradiography in the latter half of the last century, the long held assumption of cortical cell migration was tested. It became clear that post-mitotic cells attach themselves to specialised glial cells termed radial glia (because their processes radiate outwards from the ventricular zone to the outer cortical layers)(Rakic 1972; 1988). These cells provide the scaffold along which migrating cells can move to reach their final destination in the cortical plate (Cullican et al, 1990; Levitt & Rakic, 1980). More recently, *in vitro* and *in vivo* studies have observed small percentages of migrating cells which move tangentially within the cortical plate suggesting that modifications to the radial migration model may be needed (O'Rourke et al, 1992; 1995). Recent studies using vital dye to label cells in the striatal subventricular zone have supported this idea, as these cells were found to migrate into the neocortex and differentiate into γ-aminobutyric acid (GABA) positive interneurons (Anderson et al, 1997). Moving orthogonal to the long axes of radial glial cells, their path is unlikely to be laid out by the glial scaffold.

1.2.4 Cell fate

One general exception to the inside out rule of cortical development are the cells of the marginal zone (MZ) and subplate (SP), which are the first to differentiate (Marin-Padilla 1971; 1978). The MZ, containing Cajal-Retzius cells, goes on to form layer one. Cells of the SP die after thalamo-cortical innervation and are thought to play a role in this process (Allendoerfer & Shatz, 1994). The techniques of cell marking and transplantation have been used to address how and when cell fate is determined in the

cortex. In one set of experiments, cells from a donor in which deep layers were being born were labelled in S phase, and transplanted into a host where superficial layers were being born. Where they were transplanted to the ventricular zone of host animals soon after labelling, the cells took on the fates of their neighbouring cells in the host indicating that they were multipotent initially and then received instructions from the host. However, when the cells were labelled and allowed to go beyond S phase before transplantation, these cells subsequently migrated to a laminar position corresponding to their birthdate in the donor animal, indicating that they were preinstructed regarding their fate. In other words newly born cells are instructed as to their fate by environmental cues after they are born (McConnell 1988; McConnell 1991; McConnell & Kaznowski, 1991).

In more recent experiments to investigate the role of extracellular signals in regulating the differentiation of cortical cells, a cortical slice overlay assay was used in which cortical progenitor cells were cultured over cortical slices from different developmental stages. The results showed that embryonic cortical progenitors cultured over embryonic cortical slices differentiate into neurons and those cultured over postnatal cortical slices differentiate into glia. In contrast, postnatal progenitor cells differentiated into glial cells when cultured over either embryonic or postnatal cortical slices. These results suggest that the fate of embryonic progenitors can be influenced by developmentally regulated signals and that the postnatal cortex produces a diffusible factor that induces progenitor cells to adopt glial fates at the expense of neuronal fates (Morrow et al, 2001).

1.3 Patterning of the anterior neural tube and signalling centres

Prior to the influences of activity and axonal inputs (eg. from the thalamus to the cortex), the brain can be divided up into a number of units. Brain segmentation was first postulated at the end of the 19th century by Kupffer (1906), emphasizing observable transverse (neuromeric) segments in the embryonic brain, correlative with segments in the head and body of vertebrates and invertebrates. During the first quarter of the 20th century this theory was replaced in favour of a model based on longitudinal columnar arrangements of functional units, the 'columnar model' (Kuhlenbeck 1973). With the subsequent collection of large data sets for anatomy and a revised longitudinal axis, it is now recognised that transverse subdivisions are compatible and necessary in accompanying longitudinal subdivisions. Significantly, more recent data on gene expression, and corresponding functional studies, have led to a revised model, called the prosomeric model (Rubenstein et al, 1994).

This process of anterio-posterior (AP) and dorso-ventral (DV) patterning begins very early in neural plate formation, triggered at short range by morphogens diffusing from the node (Shawlot & Behringer, 1995; Shimamura et al, 1995; Acampora et al, 1996; Epstein et al, 1997; Shimamura & Rubenstein, 1997; Rubenstein & Beachy, 1998; Acampora et al, 2000). Topologically, this can be seen as specification of concentric rings of tissue. Subsequent elongation of the neural plate then introduces bilateral symmetry for all brain regions caudal to the rostral most forebrain (Rubenstein et al. 1998). AP patterning leads to major brain parts (tagma), secondary organisers (such as the isthmus and zona limitans), segmentation inside tagma (neuromeres), and general

transverse regionalisation (Doniach et al. 1992), whilst DV patterning causes longitudinal zonation. 'Primary organisers' such as the pre-caudal plate are specialised domains of tissue which express genes that induce the formation of large histogenic regions such as the forebrain. Additional levels of regional complexity are then generated by 'secondary organisers' such as the anterior neural ridge which further help to specify arealisation through more local signalling mechanisms (Shimamura and Rubenstein,1997).

An overview of the known organising centres present at E12 is illustrated in Figure 1.5. Axial mesoderm underneath the floor plate (notochord and prechordal plate (Pcp)) continues as a source of ventralising signals, secreting sonic hedgehog (Shh) protein which produces strong vertical induction effects on the overlying neural tube floor and induces the expression of a number of other genes such as *Nkx2.1* and *Nkx 2.2* which direct ventral specification and cell fate (Dale et al, 1997; Pera & Kessel, 1997; Ericson et al, 1995; Qiu et al, 1998). Concurrently, the roof plate of the closed neural tube continue to express transcription factors such as Gli3 and induces the expression of other genes (Grove et al, 2000). The roof plate expresses the homeobox gene *Lhx2* which is necessary to produce cortical ventricular zone progenitor cells (Monuki et al, 2001). The roof plate also expresses bone morphogenic proteins (BMPs) involved in cell proliferation and differentiation, and *Emx* genes, known to specify histogenic domains (Frantz 1994; Furuta et al, 1997; Liem et al, 1995; Pellegrini et al, 1996; Yoshida et al, 1997; Smith et al, 1998; Cecchi & Boncinelli, 2000). Rostrally, the anterior neural ridge secretes polypeptides of the fibroblast growth factor family (FGF) which induce the

expression of *Foxg1* in the telencephalon, driving cell proliferation (Shimamura & Rubenstein, 1997; Houart et al, 1998), and more caudally the isthmus organizing centre expresses *Wnt1* and *FGF8*, necessary for generating the cerebellum and posterior midbrain structures which develop either side of the isthmus (Xuan et al, 1995; Crossley et al, 1996; Wurst & Bally-Cuif, 2001).

Recently, the transcripts of seven members of the *BMP* family, homeobox genes *Msx1*, *Msx2* and five members of the *Wnt* gene family: *Wnt2B/3A/5A/7B* and *Wnt8B,* have been located in the medial wall of the telencephalon in an area which has been termed the *Wnt* rich cortical hem (Grove et al, 1998). As mentioned above, this area gives rise to the choroid plexus, medio-dorsal neocortex and hippocampus, and may form a local signalling centre. Indeed, knockout studies have shown that one of these genes, *Wnt3A*, is essential for the formation of the hippocampus (Lee et al, 2000).

The expression domains of some of the genes mentioned above along with many others preferentially relate to transverse limits, or coincide with longitudinal zonal limits (Puelles & Rubenstein, 1993; Martinez & Puelles, 2000). These diverse transcription factors and signals, mediating intercellular communication, presumably play on available gene enhancers to select further genes for activation or suppression. In this way specific programs of histogenesis can be initiated in each molecularly distinct part of the neural wall such that, finally, radial patterning (comprising locus-specific neuroepithelial histogenesis and stratification of diverse types of postmitotic neurons in the mantle layer) can occur with progressive maturation.

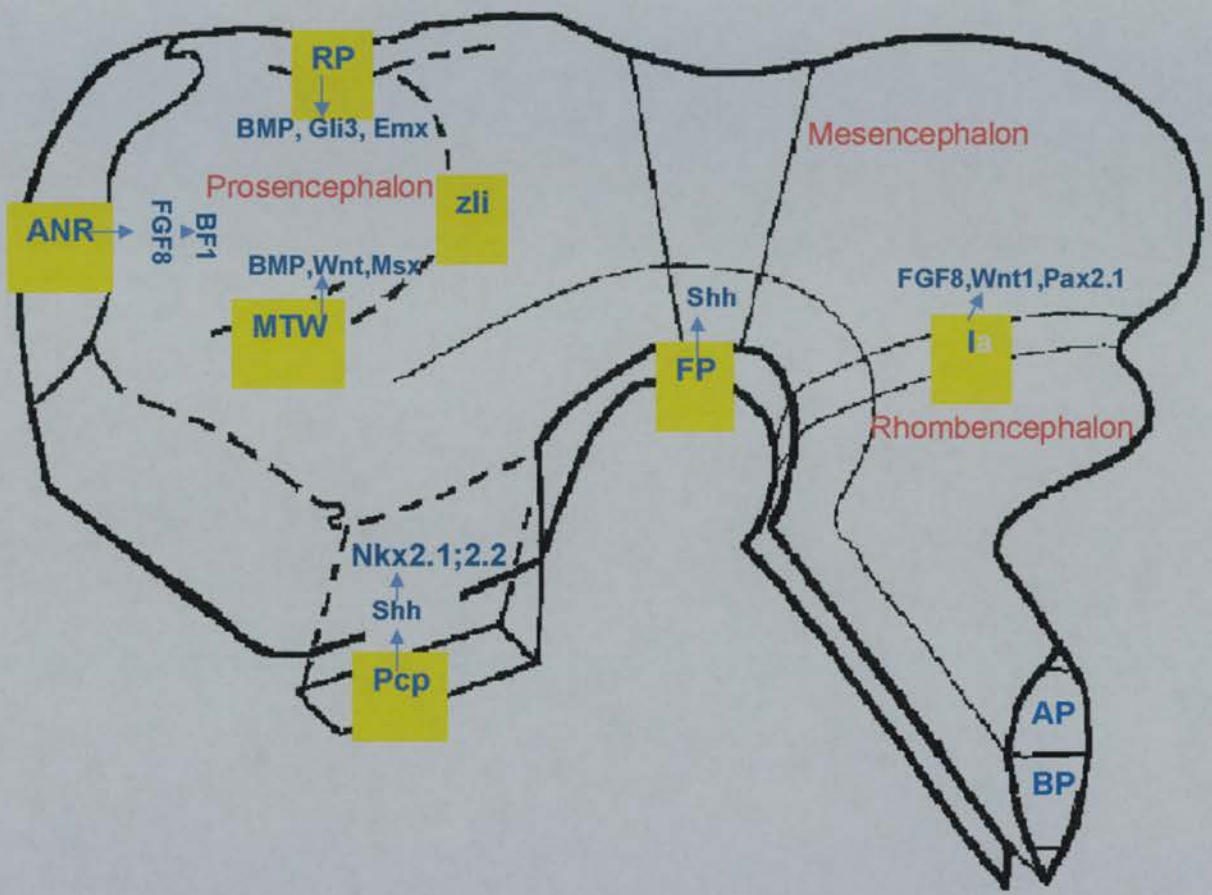

Figure 1.5 Diagram illustrating the known patterning centres in the developing brain at E12. The anterior neural ridge (ANR), roof plate (RP), floor plate (FP), isthmus (I), and pre-cordal plate (Pcp) express a number of transcription factors and signalling molecules which influence regional tissue specification and cell fate. The alar plate and basal plate are denoted by AP and BP respectively. Recently, the medial wall of the telencephalon (MTW) has been found to express members of the BMP, Wnt and Msx gene families. These factors may contribute to the development of the choroid plexus, hippocampus and dorso-medial cortex which emerge from this presumptive epithelium. Key transcription factors and signalling molecules expressed in each signalling centre are shown in dark blue against a yellow background.

1.4 The Wnt gene family

The Wnt family comprises one of the major families of developmentally important genes. The first member of this family to be identified, *Wnt1*, was discovered by Nusse and Varmus (1982), as a proto-oncogene that leads to mammary tumours in the mouse when inappropriately expressed. Building on this work, related genes were found to have a wide range of functions during embryogenesis. Processes involving *Wnt* genes include: somitic development (Takada et al, 1994; Yoshikawa et al, 1997), mesenchymal-epithelial interactions (Patapoutian et al, 1999; Stark et al, 1994), limb development (Parr & McMahon, 1995), sexual development (Vainio et al, 1999), craniofacial development (Brault et al, 2001), cell fate and proliferation (Czyz & Wobus, 2001; Yamaguchi et al, 1999; Moon et al, 1997) and development of the CNS (see below).

Wnt genes have been highly conserved over evolution and are found in a wide range of vertebrates. Nineteen members occur in humans and eighteen members have been identified in the mouse. They encode secreted glycoproteins of between 350 and 380 amino acids in length, including a conserved pattern of 23-24 cysteine residues. *Wnt* genes have been classified into two groups; the *Wnt1* class, including: *Wnt1/3/3A/8*, and the *Wnt5A* class, including: *Wnt4/5A* and *Wnt11*. This classification is based on assays carried out using mammary cell lines and Xenopus embryos. Over-expression of the *Wnt1* class was found to induce morphological transformations in some mammary epithelial cells and axis duplication in Xenopus embryos, whilst the *Wnt5a* class was found to alter morphogenetic movement during gastrulation, when ectopically expressed

in Xenopus embryos (Wong et al, 1994; Du et al, 1995). These effects were not always clear cut however (*Wnt2* and *Wnt7b* for example, have intermediate effects) (Torres et al, 1996), and this suggested that more than one signalling pathway may be involved.

Many *Wnt* genes may be classified into subfamilies based on sequence homologies consisting of a pair of more closely related genes, for example *Wnt8A* and *Wnt8B*. Whilst most *Wnt* genes share an homology of around 40%, pairs typically have >80% homology (Cadigan & Nusse, 1997).

Secreted Wnts have been shown to require sulphated proteoglycans during signalling (Lin et al, 1999) which act as co-receptors in presenting Wnt to high affinity Frizzled receptors (see below). These receptors are able to transduce Wnt signalling by the activation of up to three signalling pathways which are described below (see Figure 1.6).

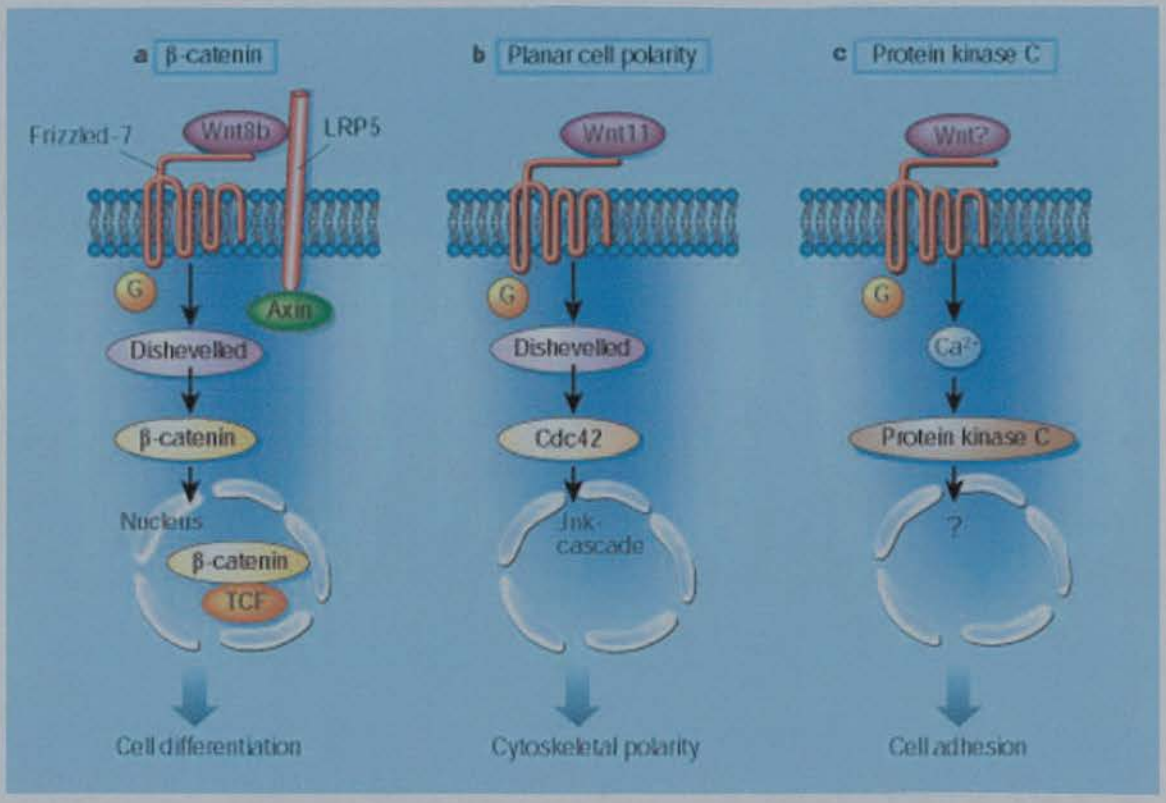

Figure 1.6 Diagram illustrating the major components of the three signalling cascades which are triggered by Wnt proteins in vertebrates (A) Canonical, or ß-catenin pathway, (B) Planar cell polarity pathway, (C) Protein kinase C pathway. Reproduced from Niehrs (2001). See text for abbreviations and explanation.

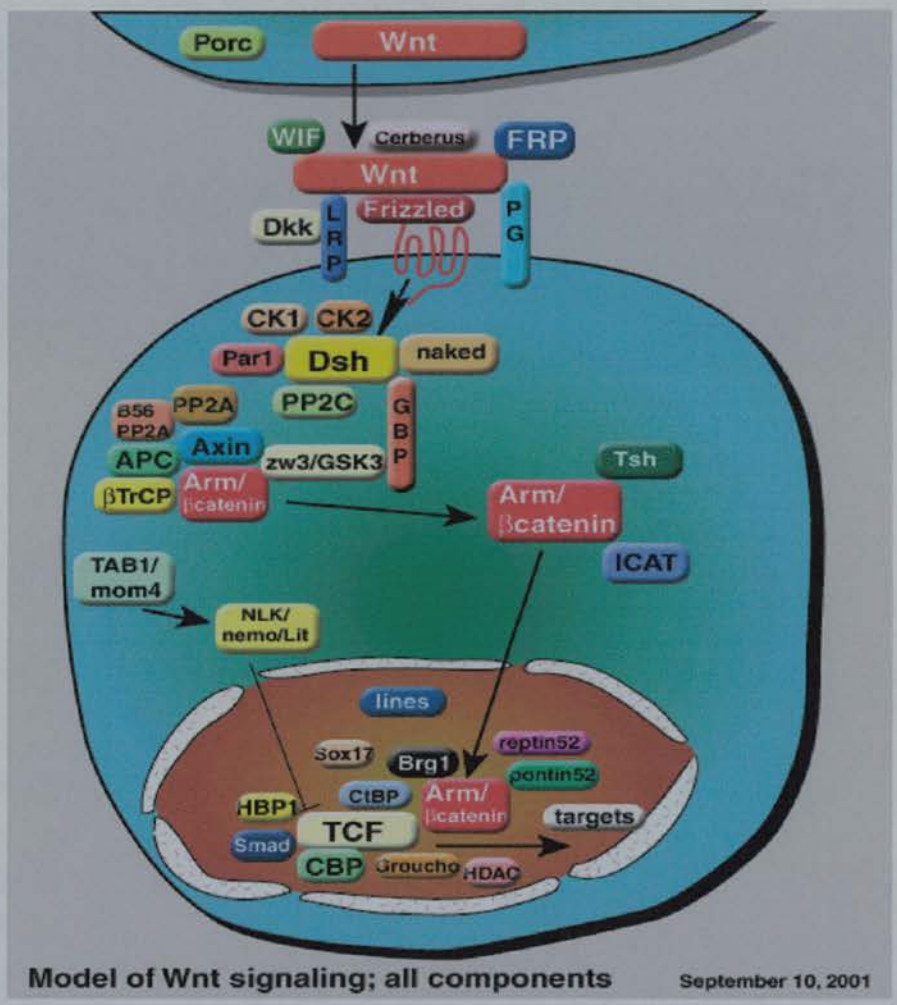

Figure 1.7 Diagram illustrating all known components of the canonical β-catenin Wnt signalling pathway. The Wnt ligand binds to the Frizzled receptor with the potential involvement of a number of co-factors. This interaction leads to the stabilisation of cytosolic β-catenin which enters the nucleus and activates transcription of target genes. In the absence of Wnt signalling, β-catenin is phosphorylated by glycogen synthase kinase3β (GSK3β) in a multiprotein complex, leading to its degradation in proteasomes. Reproduced from the Wnt homepage, http://www.ana.ed.ac.uk/rnusse/wntwindow.html Roel Nusse, Stanford University. See text for abbreviations and detailed explanation.

1.4.1 Frizzled receptors and the planar cell polarity pathway

Frizzled genes (*Fz*) encode seven-transmembrane proteins with an amino-terminal cysteine-rich domain that binds Wnts with high affinity (Alder et al, 1990; Hsieh et al, 1999). Similar to the Wnt family, multiple members of the *Fz* family have been identified (10 murine *Fz* genes have been described to date). Frizzled genes are expressed widely during development (Borello et al, 1999; Kim et al, 2001). They are imlicated in the three Wnt signalling pathways (see Figure 1.6), and are involved in many developmental processes such as craniofacial development (Sarker & Sharpe 2000), neural crest development (Deardorff et al, 2001) and cerebellar development (Wang et al, 2001).

The drosophila segment polarity gene *Wingless* (*Wg*) is the best understood Wnt family member and has been used as a paradigm in elucidating *Wnt* gene function (Rijsewijk et al, 1989). Wingless has been identified as the orthologue of Wnt1 (Cabrera et al, 1987; Rijsewijk et al, 1987) due to the identical nature of the amino acid sequences (53%) and the 23 conserved cystienes present in both genes. The identity of Wnt receptors remained elusive for some time. The first insights into understanding the mechanism of Wnt signal transduction came from a number of fly genes with mutant phenotypes consistent with defects in *Wg* signalling. Drosophila mutations in the first *Fz* gene were found to display a mis-alignment of epithelial cells which caused disruption in the orientation of wing hairs and uncovered the existence of the planar polarity pathway (PCP)(Vinson & Alder, 1987). *Fz* mutants were also found with similar effects on leg bristles, and the ommatidia of the compound eye (Alder 1992; Zheng et al, 1995), a

phenotype shared with several other mutations including dishevelled (Dsh) (Theisen et al, 1994; Krasnow et al, 1995). This raised the possibility that *Fz* molecules were involved in Wnt reception, and subsequently, Dfz2 was found to confer on cultured cells the ability to bind and respond to Wg (Bhanot et al, 1996). Soon afterwards, *Fz* homologues were found to function in Wnt-mediated signalling in vertebrate tissues, with mutant phenotypes similar to those found in *Wnt* mutants (Yang-Snyder et al, 1996; He et al, 1997; Tan et al, 2001; Wang et al, 2001).

The major components of the PCP pathway are illustrated in Figure 1.6b. Binding by an appropriate Wnt ligand leads to the activation of GTP-binding proteins from the Cdc42/Rho family which activate the transcription factor Jun. Frizzled receptors are also implicated in the other Wnt signalling pathways (see Fig1.6).

1.4.2 The Wnt/β-catenin signalling pathway

The best characterised signalling pathway triggered by Wnt proteins (through binding to Frizzled receptors) is the Wnt/β-catenin pathway (Figure 1.6a, 1.7). Details of this pathway also first emerged from genetic analysis of Drosophila where it functions in developmental processes such as patterning of body segments and appendages (Noordermeer et al, 1994; Peifer et al, 1994; Siegfried et al, 1994). Intracellularly, the state of stability of the cytoplasmic pool of β-catenin (a second pool is associated with cadherins at the cell surface) provides the critical element in the transduction of the Wnt signal.

In the absence of Wnt, the pool is low due to targeted degradation of β-catenin which is phosphorylated by glycogen synthase kinase-3β (GSK3β) in a multiprotein complex. Upon Wnt signalling, dsh blocks β-catenin degradation (possibly via the GSK3β inhibitor GBP) which leads to the stabilisation of β-catenin, and consequently it can be translocated to the nucleus where it interacts with members of the Tcf family of transcription factors and leads to transcription of target genes (Filali et al, 2002). A number of target genes have been identified and include developmental regulatory genes such as *twin* in Xenopus (Laurent et al, 1997) and *ultrabiothorax* in Drosophila (Riese et al, 1997). Additional targets include c-myc and cyclinD1 which are regulators of cell growth and proliferation (Tetsu & McCormick, 1999; He et al, 1998). In the absence of β-catenin Tcf proteins mediate repression when bound to members of the Groucho family of transcriptional repressors, and CtBP (Roose & Clevers, 1999).

Recent studies have uncovered an increasing number of proteins that play a role in regulating β-catenin stability. The proteins Axin, adenomatous polyposis coli (APC) and glycogen synthase kinase-3β (GSK3β) directly promote the degradation of β-catenin. These proteins form a complex which functions to maintain a low steady state of β-catenin in the cell. Axin and APC serve as scaffolds binding both β-catenin and GSK3β facilitating the phosphorylation of β-catenin by GSK3β and its subsequent degredation. Mutation of zeste-white 3 (zw3), the Drosophila orthologue of GSK3β results in accumulation of Armadillo, the Drosophila orthologue of β-catenin, and phenotypes that

mimic constitutive activation of the Wnt/β-catenin pathway (Peifer et al, 1994; Siegfried et al, 1994; Dierick & Bejsovec, 1999). β-catenin also binds to cadherin cell adhesion molecules providing a link to the actin cytoskeleton (Taipale & Beachy. 2001; Sharp et al. 2001; Zorn 2001; Giarre et al. 1998; Cadigan & Nusse. 1997).

1.4.3 The Wnt/ Ca^{2+} signalling pathway

The cellular responses of transformation and morphogenic movement in Xenopus embryo's, used as the basis of the activity-based classification system of Wnt genes (as described in section 1.4) attribute these responses to the activation of the canonical Wnt/β-catenin pathway. More recently, however, the non-transforming Wnt5A class have been found to elicit Ca^{2+} release and to activate CamKII and Protein kinase C (PKC) in a G-protein dependent manner (Slusarski et al, 1997,1997b; Sheldahl et al, 1999) (Figure 1.6c). Hence, the Ca^{2+} release may be implicated in these responses.

Under some circumstances Wnts may couple to both pathways, suggesting distinct cellular responses depending on which receptors are present (He et al. 1997). It had been found that rat Frizzled1 but not rat Frizzled2, induces β-catenin target gene expression in Xenopus assays (Yang-Snyder et al, 1996). In zebrafish, rat Frizzled2 induced intracellular calcium release to a greater extent than rat Frizzled1 (Slusarski et al, 1997). These data were interpreted to indicate that Frizzled receptors may preferentially stimulate either β-catenin or the activation of Ca^{2+} and PKC. Further work was carried out using the induction of Wnt/β-catenin target genes and PKC activation as assays for

Frizzled activity in Xenopus. The results demonstrated that there are two functionally distinct classes of Frizzled proteins. Expression of rat Frizzled1, mouse Frizzled7/8, Xenopus Frizzled1 and Drosophila Frizzled and Frizzled1 activated β-catenin targets but not PKC. Conversely, expression of rat Frizzled2 and mouse Frizzled3/4/6 activated PKC but not β-catenin targets (Sheldahl et al, 1999). Importantly, all three signalling pathways described above have recently been shown to be activated through one Frizzled receptor, Frizzled-7 (Niehrs, 2001), and this was found to be dependent upon the Wnt ligand present. In Xenopus, Wnt8B has been shown to trigger the Wnt/β-catenin pathway (Sumanas et al, 2000) and Wnt11 to activate the planar polarity pathway (Wallingford et al, 2000). Recent experiments to inhibit Frizzled-7 production using antisense oligonucleotides resulted in gastrulation defects in Xenopus embryos. This effect was reversed by injection of protein kinase C but not by components of the β-catenin or PCP pathways, showing that an unknown Wnt protein and Frizzled-7 couple to a PKC activating cascade which is distinct from the β-catenin and PCP pathways (Winklbauer et al, 2001).

1.4.4 Wnt antagonists

Wnt function can be modulated extracellularly through interaction with various secreted factors such as Wnt inhibitory factor-1 (WIF-1), secreted Frizzled related proteins (SFRPs), and the proteins Cerebrus and Dickkopf (Dkk). These factors have been found to inhibit the activity of Wnt proteins, predominantly by blocking their interaction with Frizzled proteins (Brown & Moon, 1998).

The protein WIF-1 has been shown to bind to Drosophila Wg and Xenopus Wnt8, inhibiting their activity (Hsieh et al, 1999). SFRPs, a group of putative Wnt antagonists contain a cysteine rich domain, homologous to that found in the Frizzled receptors and have also been shown to bind to Wnts and inhibit their activity. SFRP-5 for example has been shown to inhibit the ability of Xwnt8 mRNA to induce axis duplication in Xenopus embryos (Chang et al, 1999). Six SFRP family members have been identified in human, mouse and chick (Lescher et al, 1998; Xu et al, 1998; Ladher et al, 2000). Cerebrus is a secreted factor expressed in the Xenopus gastrula organiser. In early Xenopus embryos it has also been shown to inhibit Wnt signalling and is proposed to act here to sharpen the boundries of cell fate (Glinka et al, 1997; Brown & Moon, 1998). A Drosophila knockout of the gene *Arrow* posesses an identical phenotype to Wg mutants. *Arrow* encodes a transmembrane protein that is homologous to two members of the mammalian low-density lipoprotein receptor (LDLR)-related protein (LRP) family which have been shown to function as co-receptors for Wnt signal transduction and may form part of the Wnt receptor complex (Pandur & Kuhl, 2001). Dickkopf (Dkk) antagonises Wnt action by binding LRPs and preventing the formation of active Wnt-Frizzled-LRP receptor complexes (Bafico et al, 2001).

Such interactions, with multiple inhibitory factors may act to fine tune the spatial and temporal patterns of Wnt activity by modulating Wnt signalling generally, or specific ligand-receptor relationships which may in turn influence the choice of signalling pathway activated.

1.4.5 *Wnt* and *Frizzled* genes in the CNS

Many members of the Wnt and Frizzled families are expressed in the developing CNS and perform roles in the regulation of cell proliferation, cell fate, programmed cell death, synaptogenesis, patterning, and in the formation of forebrain structures such as the hippocampus.

Evidence for the involvement of *Wnt* genes in proliferation in the CNS came from mice with compound mutations in both *Wnt1* and *Wnt3A* which are co-expressed in the dorsal midline of the developing neural tube. In the hindbrain of $Wnt1^{-/-}Wnt3A^{-/-}$ animals there was a pronounced reduction in the numbers of dorsolateral neural progenitors which was not observed in single mutants of either gene alone. This suggested that Wnt signalling regulates the expansion of these cells either as a survival factor or a mitogen (Ikeya et al, 1997). The latter possibility was supported by transgenic analysis which demonstrated that Wnt1 can act as a potent mitogen when ectopically expressed within the dorsal CNS (Dickinson et al, 1994). More recently, a mutation in *Pax-6* (a homeobox gene, necessary for regulating the development of the diencephalon) was found to cause abnormally low proliferative rates during early mouse development (Warren & Price, 1997). Expression studies of *Wnt7B* and *SFRP2* in the *Pax-6* mutant suggest that changes in the signalling of these factors may contribute this phenotype (Kim et al, 2001). Taken together, these observations suggest that one role of Wnt genes in the CNS involves the stimulation of proliferation.

Wnt signalling is also known to influence cell fate. In the chick embryo, continual Wnt signalling has been found to block the response of epiblast cells to FGF signals, permitting the expression and signalling of BMP to direct an epidermal fate. Conversely, a lack of exposure of epiblast cells to Wnt signals was found to permit FGFs to induce a neural fate (Wilson et al. 2001). In stem cells, interaction with the extracellular matrix via integrins was found to determine the expression of BMP4 and Wnt1, resulting in the activation of mesodermal and neuroectodermal lineage respectively (Czyz & Wobus, 2001). Evidence for the involvement of Wnts in programmed cell death and synaptogenesis also exists. In the avian hindbrain the overexpression of SFRP2 has been found to inhibit programmed cell death (Ellies et al. 2000), and Wnt 7A expressed by cerebellar granule cell neurons has been found to regulate the presynaptic differentiation of mossy fibres (Hall et al, 2000).

Indications for a role of *Wnt* genes in midbrain-hindbrain patterning came from studies of mice in which the *Wnt1* allele was inactivated (McMahon & Bradley, 1990). In these animals the midbrain and some rostral metencephalon were absent. Additionally, the deletion of a single base pair from *Wnt1* (predicted to cause premature termination of translation) was found to disrupt development of the cerebellum (Thomas et al, 1991). Patterning (abnormal cerebellar foliation) was also found to be disrupted in mice with null alleles of *Engrailed-2*, a molecular homologue of the Drosophila gene *engrailed* (Kuemerle et al, 1997), and a transcriptional target activated by Wnt1 signalling (McGrew et al, 1999). It has been shown that multiple *Wnt* genes are expressed in a

restricted domain of the medial wall of the developing cerebral cortex, the cortical hem, and this suggests *Wnt* genes may also play an important function in forebrain development. Expression of *Wnt2B/3A/5A* and *Wnt8B* was found in overlapping patterns together with homeobox genes of the *Msx* family and a number of members of the BMP family of morphogenic proteins (Grove et al. 1998). These data indicated that this area may form a regional patterning centre which could play a role in forebrain development. This was confirmed in mice lacking *Wnt3A* which exhibited a general failure of proliferation within the medial neuroepithelium and failed to form a hippocampus (Lee et al, 2000). In the same year, analysis of mice containing a *Lef1* null mutation found a dramatic reduction in the number of dentate gyrus granule cells whilst the remaining dentate gyrus cells and hippocampus remained intact. *Lef1* is a member of the LEF1/TCF family of transcription factors mediating Wnt signalling. Mice carrying a dominant negative mutant allele of *Lef1* which also interfered with β-catenin mediated transcriptional activation by other LEF1/Tcf proteins, was found to lack the entire hippocampus, mimicking the *Wnt3A*$^{-/-}$ phenotype (Galceran et al, 2000).

Additionally, the expression of a number of Wnt receptors, Fz5/8/9/10, and the secreted Frizzled related proteins SFRP-1 and SFRP-3 have also been found in the cortical hem in gradients of expression (Kim et al, 2001). Taken together, these data strongly suggest that *Wnt* genes play an important role in the development of the medial telencephalic wall.

Recently, β-catenin gene deletion in the region of Wnt1 expression has been found to disrupt midbrain and cerebellar development in mice (Brault et al, 2001), and in Xenopus embryos, a gradient of Wnt/β-catenin signalling has been found in the presumptive neural plate indicating that it acts in early CNS patterning (Kiecker & Niehrs, 2001). Further, studies in zebrafish have found that interference with Wnt8 translation abrogates formation of the spinal cord and posterior brain fates (Erter et al, 2001), and a mutation in the GSK3β binding domain of *masterblind/Axin1* severely disrupts forebrain development in zebrafish (Heisenberg et al, 2001).

Taken together these data show that signalling by multiple *Wnt* genes is nesessary in the normal patterning and development of the CNS, and further, that the roles played by these factors is at an early stage of understanding.

1.5 Proteoglycans and heparan sulphate proteoglycans

Proteoglycans (PGs) are abundant cell-surface molecules expressed by most animal cells. They consist of glycosaminoglycan (GAG) polysaccharides covalently linked to a core protein. PGs can be associated with the extracellular matrix or cell surface, and have been implicated as co-factors in processes ranging from mechanical support to functions in adhesion, motility, proliferation, differentiation, morphogenesis and thalamocortical development (Bernfield et al, 1999; Kinnunen et al, 1999; Baeg & Perrimon, 2000; Selleck, 2000). Historically, it was thought that these molecules acted

as general low affinity receptors aiding cell-cell interaction. Recent studies, though, are revealing selective effects of these molecules.

1.5.1 Proteoglycan synthesis and structure

The functional specificity of proteoglycans is most likely due to their structural heterogeneity, which is conferred in part by their core protein, and in part by the species of GAG attached to it, and the modification of this chain during biosynthesis. The biosynthetic pathway of PGs is illustrated in Figure 1.8.

Initially, in the Golgi, a common tetrasaccharide linkage region is transferred to the core protein at specific attachment sites containing Ser-Gly residues (of which there may be up to 5 on each core protein). The chain is then built up by alternating the addition of an aminosugar and glucuronic acid (GlcA), and undergoes sequential modification by Golgi enzymes; N-deacetylase/N-sulphotransferase, GlcA C5-Epimerase (converting GlcA to iduronic acid), and 2-,6- and 3-0-sulphotransferase, which may add sulphate groups to the 2,3 and 6 position of the saccharide units (Perrimon & Bernfield, 2000; Prydz & Dalen, 2000). The GAG species (of which there are five) is determined by the addition of the fifth disaccharide (Fritz et al, 1997)(see Figure 1.8), and subsequent modification of the polymerised chain by Golgi enzymes. Heparin can be distinguished from Heparan Sulphate Proteoglycans (HSPGs), a structurally similar related proteoglycan, by the extent of epimerisation of glucuronic acid to iduronic acid, and by the sulphation pattern of the disaccharide units (Habuchi et al, 1998). The expression of HSPGs can be seen in almost all cell types, whilst the expression of heparin is mainly confined to connective

tissue mast cells. Another distinguishing feature between the two proteoglycans is that heparin is largely concentrated in the intracellular portion of cells, whilst HSPGs are generally targeted to the cell surface and extracellular matrix (Bullock et al, 1998; Binari et al, 1997).

Generally a higher level of organization of of each glycosaminoglycan chain is also constructed, with 10-16 highly modified dissacharides alternating with longer, relatively unmodified dissacharides in a chain size which can vary from 20 to 150 dissacharides in length. Tissue specific isoforms of the polymerising and chain modifying enzymes are also known, and produce chains with distinct sequences and topographical organizations (Habuchi et al, 1998). Targeting of PGs to specific cellular locations is achieved through recognition of core proteins which can be: transmembrane (syndecans), plasma membrane linked (glypicans), or secreted into basement membranes (perlecan, agrin)(see Figure 1.9). Although syndecans and glypicans are associated with the plasma membrane, the core proteins can be proteolytically cleaved near the cell surface releasing the intact ectodomain together with all the HS chains of the parental molecules (Bernfield et al, 1999). In addition to the diversity of the core proteins of the PGs, each protein has a distinctive tissue specific expression pattern, cellular localisation, and may perform functional roles. For example; syndecans have been found to affect morphology and migration (Longley et al, 1999; Ethell & Yamaguchi, 1999) and glypicans to affect axon guidance, cell division and TGF-β/BMP activity (Jackson et al, 1997; Liang et al, 1999; Ronca et al, 2001).

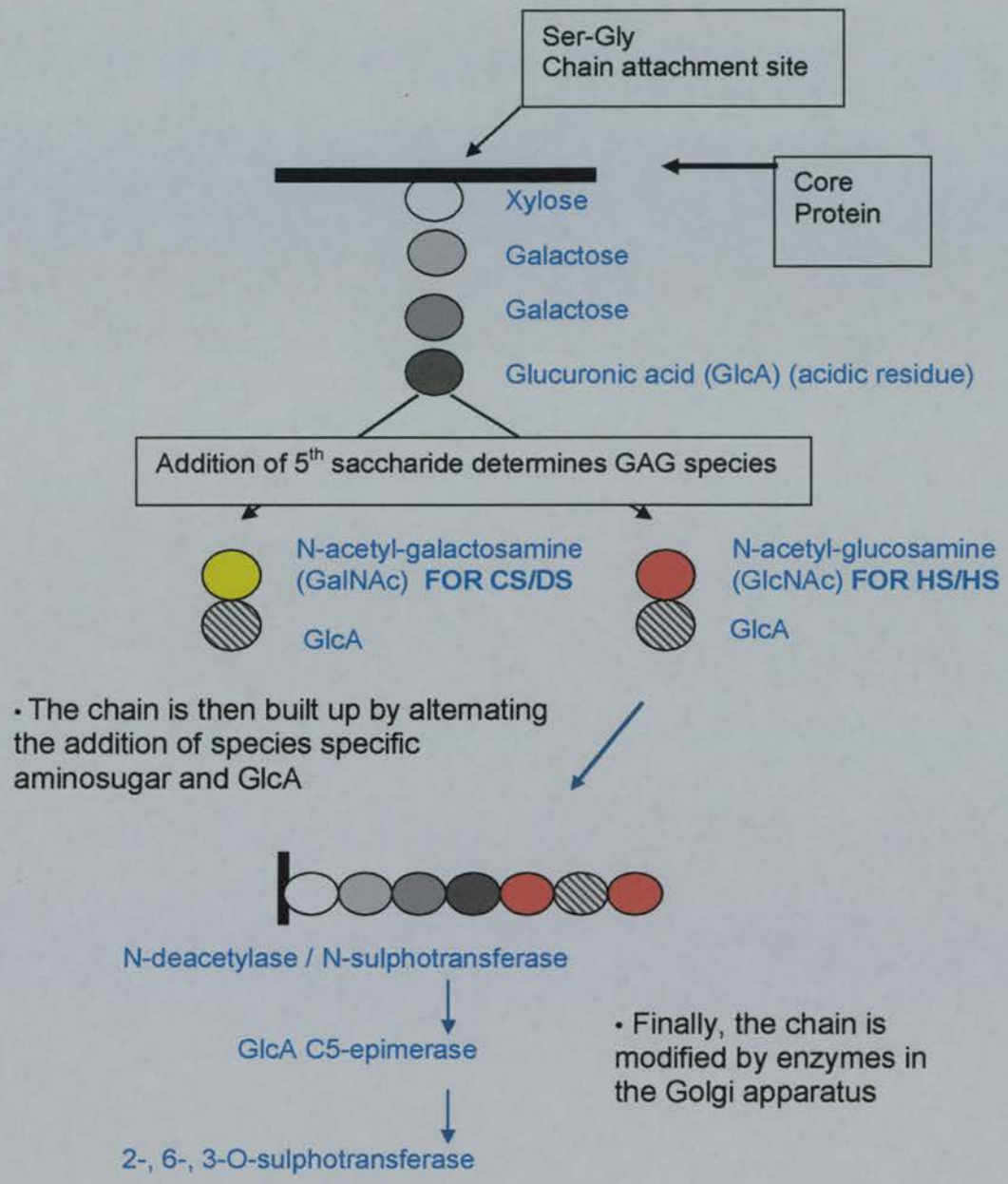

Figure 1.8 Schematic diagram of proteoglycan synthesis. From attachment sites on a core protein, a glycosaminoglycan chain is constructed. The composition of this chain determines the species of glycosaminoglycan produced. Subsequently, the chain is modified by a number of enzymes in the Golgi apparatus.

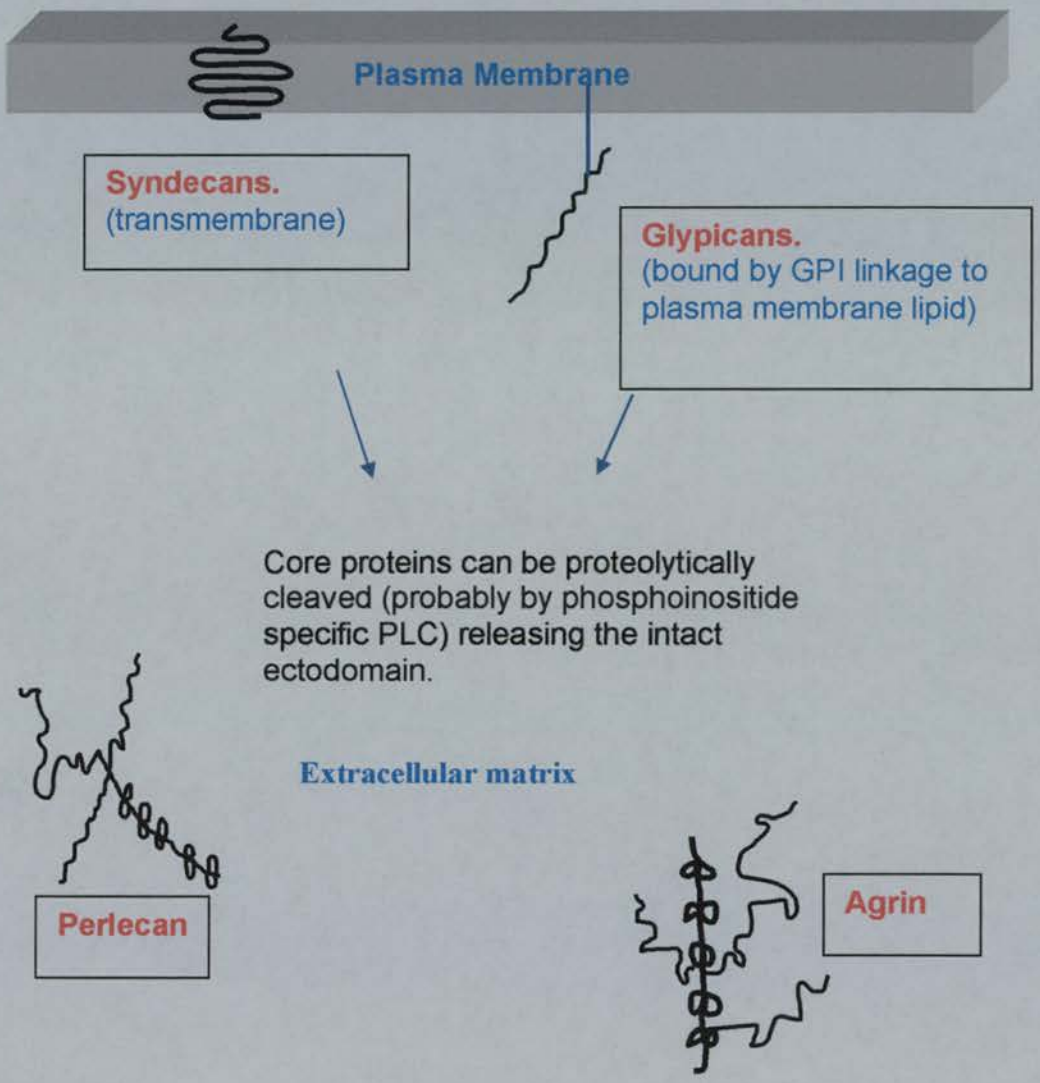

Figure 1.9 Schematic diagram of proteoglycan localisation. After translation and modification of the proteoglycan in the Golgi apparatus, the species of core protein determines its cellular/extracellular location. Syndecans and glypicans are membrane bound whereas agrins and perlecans are secreted into the extracellular matrix.

1.5.2 Heparan sulphate proteoglycans and development

The importance of HSPG molecules in developmental processes and specific signalling pathways has been illustrated by the identification of mutations in enzymes involved in heparan sulfate (HS) chain biosynthesis in Drosophila and mice (Bellaiche et al, 1998; Lin & Perrimon, 1999), and by *in vitro* studies (Reichsman et al, 1996). These indicate that HSPG molecules are required in the signalling pathways of FGFs (Rapraeger et al, 1991), Wnts (Itoh & Sokol, 1994), hedgehog (Bellaiche et al, 1998), and BMPs (Paine-Saunders et al, 2002). Additionally, studies of the sulphation patterns of HSPGs have defined motifs required for specific interactions with growth factors, cytokines, matrix components, enzymes and other molecules (Salmivirta et al, 1996). Loss of the gene *Sulfateless* in Drosophila, which encodes N-deacetylase/N-sulphotransferase and is important in post-translational modification of HSPGs has been found to affect *Wingless* signalling (Lin & Perrimon, 1999).

One of the most striking examples of the role of HSPGs during development comes from a gene trap mutation in the murine gene encoding heparan sulphate 2-sulphotransferase (*Hs2st*), an enzyme which adds a sulphate molecule to the 2-0 position of heparan sulphate (Bullock et al, 1998).

Gene-trapping methodology is a powerful strategy for cloning and identifying functional genes. Various trapping strategies allow genes to be segregated based on criteria like the specific subcellular location of an encoded protein, the tissue expression profile, or responsiveness to specific stimuli. Integration into the genome (of murine ES cells by

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electroporation) of gene trap vectors provide specific sequences that generate fusion RNA transcripts when inserted into a gene. These can then be identified by mRNA sequence (Stanford et al, 2001).

Homozygous mutant *Hs2st* animals display a number of developmental abnormalities and do not to survive past birth, which is most likely due to bilateral renal agenesis. Homozygous mutants also exibit defects in the eye and skeletal development.

In this mutant, *LacZ* reporter gene activity was used to determine the expression pattern of *Hs2st* in heterozygous embryos. This revealed expression in all three germ layers at E7.5. By E10.5, expression was found to be strongest in the dorsal and ventral aspects of the neural tube, brain and midbrain-hindbrain junction. A day later, expression was strongest in the floor plate and roof plate, the mesenchyme of the limb and throughout the cerebral cortex. No expression was observed in the dorsal thalamus. By E13, expression occurred in the developing teeth and whisker follicles (Bullock et al, 1998). As mentioned, HSPGs are necessary in a number of signalling pathways and it is likely that the phenotype displayed by $Hs2st^{-/-}$ animals is the result of interference in one or more of these.

Heparan sulphate proteoglycans and FGF signalling

In 1991 it was found that FGF-2 could neither bind to its high affinity receptor (FGFR-1) nor exert its proliferative or anti differentiative effects on cells deficient in sulphated proteoglycans (Rapraeger et al, 1991; Yayon et al, 1991). In a separate study examining

a related molecule (FGF-1), it was found that one part of the HS molecule binds to FGF-1 and another to FGFR-1, such that a ternary complex is formed before signal transduction can occur (Ornitz et al, 1992). Morover, the requirement for heparan sulphate is a property of all FGFRs (Mansukhani et al. 1992; Ornitz & Leder. 1992). Subsequently, it was found that FGF1 and a related molecule, FGF2 are recognised by different HS structures expressed in discrete domains of the HS polymers. *In vitro* experiments have revealed that N-sulphation is necessary for high-affinity binding of FGF2 (Ishihara et al, 1992; Ishihara et al, 1993). Additionally, chlorate treatment (5-20mM) of Madin-Darby canine kidney (MDCK) cells reduces 6-0-sulphation, whereas higher concentrations (50mM) also reduce 2-0-sulphation but not N-sulphation. In parallel with the dose dependent loss of 6-0-sulphation there is a reduction in binding to FGF1, whereas binding to FGF2 remains unchanged (Kreuger et al, 1999).

Heparan sulphate proteoglycans and BMP signalling

The *Noggin* gene encodes a secreted polypeptide which binds to members of the TGF-β superfamily such as the BMPs. In a recent study, Noggin was found to bind strongly to heparin *in vitro*, and to heparan sulfate proteoglycans on the surface of cultured cells which expressed heparan sulfate (Paine-Saunders et al, 2002). Hence, interaction between Noggin and HSPGs in vivo may regulate diffusion, and therefore the formation of gradients of BMP activity during development.

Heparan sulphate proteoglycans and hedgehog signalling

Mutations in the Drosophila gene *tout velu* (ttv), which encodes a putative HS polymerase have shown that hedgehog (Hh) signalling is specifically affected in this

mutant and not Wg or FGF signalling, as observed in *Sugarless* (in which no HSPG molecules are produced) and *Sulfateless* (described above) mutants (Bellaiche et al, 1998). Further studies have suggested that the membrane targeted cholesterol modified Hh molecule requires HSPGs, either to be trapped by receiving cells or to move from cells to cells (The et al, 1999).

Heparan sulphate proteoglycans and Wnt signalling

Sulphated proteoglycans have been shown to act as co receptors for Wnts as a nesessary component of Wnt signalling. Evidence for a role of proteoglycans in Wnt signalling came from analysis of the Drosophila mutations *sulfateless* and *dally.* The Drosophila gene *sulfateless* (*sfl*) encodes a homologue of vertebrate heparan sulphate N-deacetylase/N-sulfotransferase which is necessary for HS modification, and is essential for Wg signalling. The gene *dally* (division abnormally delayed), encodes a glycosyl-phosphatidyl inositol (GPI) linked glypican, which acts as a co-receptor for *Wg* (Lin & Perrimon, 1999; Tsuda et al, 1999). Additionally, sulphated glycosaminoglycans were found to be required for soluble Wg to optimally stabilise Arm in a Drosophila cell line, and the addition of exogenous heparin was found to induce *Wg* signalling (Reichsman et al, 1996). Removal of heparan sulphate (via heparinase) has also been shown to block Wnt8 activity in Xenopus embryos (Itoh & Sokol, 1994).

In mammals, interference with proteoglycan synthesis leads to loss of *Wnt11* expression and kidney development (Kispert, 1996), and it has been found that *syndecan-1* expression is necessary for *Wnt1* induced mammary tumourigenesis in mice (Alexander

et al, 2000). Additionally, in a recent study in mice, it was found that the *Wnt*/β-catenin pathway was activated by increasing levels of extracellular proteoglycans in the epithelia of the stomach, duodenum, and jejunum, which influenced cell proliferation in this region (Perreault et al, 2001). An avian heparan specific sulphatase termed QSulf1 has been found to be localized on the cell surface in C2C12 myogenic progenitor cells and regulates heparan-dependent Wnt signaling through desulphation of cell surface HSPGs (Dhoot et al, 2001).

This evidence increasingly points to complex roles for HSPGs in these signalling pathways which are crucial for the normal development of many species. The complex nature of proteoglycans and their subtle modifications, such as the extent of sulphation, may confer specificity of interaction with the factors described above.

1.6 Cell proliferation in the adult brain

Using autoradiography, Altman was first able to observe the proliferative potential of the adult rodent brain (Altman, 1965), though the significance and existence of adult neurogenesis were controversial. In 1985 Rakic published a paper entitled 'limits of neurogenesis in primates'. Using autoradiography for tritiated thymidine injected into rhesus monkeys, he failed to find any proliferating cells in the brain and postulated that unlike non primates, neurons in the brains of primates are all generated during the prenatal period, and having a stable population of neurons was important for the continuation of learning and memory over a lifetime. Under the weight of this paper,

studies into adult neurogenesis ceased for a period of time. In 1997, BrdU positive cells were found in the brains of five patients that had been given BrdU injections to monitor cancers confined to the throat and mouth (Eriksson et al, 1998). This work renewed and redoubled research in this area providing a large weight of evidence in favour of adult neurogenesis.

In rodents, it is now clear that at least two populations of proliferating cells exist. One population is at the ventricular edge of the lateral ventricles in the cerebral cortex, and a second population is in the subgranular layer of the dentate gyrus of the hippocampus (Gould et al, 1999). Cells generated by the former population have been shown (in the rat) to migrate in the rostral migratory stream (RMS) towards the olfactory bulb where they differentiate into neurons, and cells from the latter population have been shown to migrate the short distance into the contiguous granule cell layer where they differentiate into neurons and form connections (Luskin, 1998; Palmer et al, 1997; Cameron & McKay, 2001).

Early research (using mouse and rat) aimed to discover whether these neuronal progenitors were stem cells, with a view to devising therapeutic applications. Hippocampal derived cells have been expanded *in vitro* and implanted back into the hippocampus where they have generated new neurons and glia (Suhonen et al, 1996; Palmer et al, 1997). When transplanted into the RMS they migrate to the olfactory bulb and differentiate into neurons positive for tyrosine hydroxylase, which is not produced by those in the hippocampus (Suhonen et al, 1996). *In vitro*, rat hippocampal cells have

also been induced to differentiate into excitatory (glutaminergic) and inhibitory (GABAergic) neurons under the influence of neurotrophin-3 (NT-3) and brain derived neurotrophic factor (BDNF) respectively (Vicario-Abejon et al, 2000). On the basis of this evidence it seems likely that these hippocampal progenitors are stem cells. However, some workers have found difficulties in expanding hippocampal derived cells in culture, and while this may be due to experimental conditions, the absence of a stem cell marker still leaves a question mark here. Due to this doubt the term adult hippocampal precursor cells (AHPs) will be used in this thesis.

In an attempt to address the functionality of cells derived from AHPs, Gould (1999) used BrdU labelling studies in macaque monkeys to identify new neurons in prefrontal, inferior temporal and parietal areas of the neocortex, which along with the hippocampus are also areas involved in learning and memory. Many other studies have focussed on the wide variety of factors which influence neurogenesis in the hippocampus. An enriched environment, where mice have been given access to toys and interesting objects, has been shown to increase neurogenesis (Kempermann & Gage, 1997; 1998; 1999; Nilsson, 1999; Kaplan, 2001), as did voluntary exercise (Praag et al. 1999), transient forebrain ischemia (Takagi et al. 1999), and growth factors such as epidermal growth factor (EGF), fibroblast growth factor-2 (FGF2) (Kuhn et al, 1997) and insulin-like growth factor-1 (IGF-1) (Aberg et al, 2000). Similar effects on neurogenesis have been found with antidepressants (Malberg et al, 2000; Manev et al, 2001; Michaelis et al, 2001), oestrogen levels (Tanapat et al, 1999; Ormerod & Galea, 2001), and electrically and chemically induced seizures (Scott et al, 2000; Parent et al, 1997; Grey

& Sundstrom, 1998). Factors found to decrease proliferation have included high corticosteroid levels, stress and the advancement of age (Cameron et al, 1999; Albeck et al, 1997; Gould et al, 1997,1998).

In a most recent study, newly generated hippocampal neurons have been implicated in the formation of new memories (Shors et al, 2001). In this study, rats were conditioned to associate stimuli which were presented in a temporal sequence (hippocampus dependent). A DNA methylating agent (methylazoxymethanol acetate) was then administered to reduce cell proliferation (giving an 80% decrease in the dentate gyrus). It was found that this impaired hippocampal dependent learning, and that this ability was restored on returning cell proliferation to normal levels, post drug administration. A similar reduction in cell proliferation was found not to affect learning when the same stimuli were not separated in time, a task that is hippocampal- independent (Schmaltz & Theios, 1972).

An alternative theory for the function of these cells is to repair and replace those lost in the subgranular layer of the dentate gyrus. These cells have been proposed to die due to exposure to high levels of glutamate, as this area is the first point of call for all sensory information arriving at the cortex (Gage, 2000). This theory in part rests on an assumption of the classical tri-synaptic model of the hippocampus. In this model, the first link arises in the entorhinal cortex and terminates in the dentate gyrus. The second link arises from the granule cells of the dentate gyrus and terminates on the CA3 pyramidal cells of the hippocampus. Finally, the third link arises from axons of the CA3

cells which terminate on the CA1 cells (Swanson et al, 1987). Detailed anatomical studies have shown that this model is a gross oversimplification (Amaral, 1993). Figure 1.3 illustrates how the input pathway to the hippocampus is directed to all regions of the hippocampus. If replace and repair was a necessary function of these cells, then it might be expected that new neurons should be found in the CA fields also, which is not the case to date (Gould, 1999).

As reported, many factors affecting AHP cell proliferation have been found. However, the molecular mechanisms involved in these processes are still to be identified.

1.7 Aims and objectives of this study

The main aims and objectives of this study were to use expression analysis and recently generated transgenic knockout animals as a tool to investigate the importance of Wnt signalling on brain development. There is a large volume of literature dedicated to components of the Wnt pathway during development. However, many important aspects remain to be investigated. This study attempts to address two main questions.

The first part of this study involved the phenotypic analysis of a *Wnt8B* transgenic knockout animal. Many members of the *Wnt* gene family had been found to play important roles in development. Thus far, the expression and function of this member of the *Wnt* gene family had not been thoroughly investigated.

The second stage of this study was to carry out a phenotypic analysis of an *Hs2st* knockout animal. HSPGs are known to be a necessary component of Wnt signalling in Drosophila, but less data exists in mammmals. Their complex and heterogeneic structure gives them the potential to play specific roles in growth factor signalling. The aim of this study was to examine whether a very selective modification of this molecule would have functional consequences in CNS development, possibly through an effect on Wnt signalling.

CHAPTER 2 Materials and methods

2.1 Standardisation of embryonic ages

The work described in this study involved the use of embryonic and adult mice. Staging of embryonic mice was carried out by denoting the day of vaginal plug as embryonic day 0 (E0) and counting on subsequent days from this point. However, simply adding one day (each day) to the day of vaginal plug did not consistently result in a corresponding Thieler stage (Theiler, 1989). This may have been due to individual matings taking place at at different times over night, which were up to 12 hours appart. Additionally, even within a single litter different developing stages were observed. To correct for these differences embryonic mice from E7.5 to E10.5 were reassessed after dissection on the basis of anatomical features (based on the staging system of Theiler, (see also Kaufman 1992)). E7.5 embryos were denoted by the presence of an early headfold and absence of somites; E8-8.5 by the presence of by the presence of 9-11 pairs of somites and at an early stage of turning; E9.5 by completion of the process of turning, and E10-10.5 by the emergence of the telencephalic vescicles.

2.2 Dissection and tissue preparation

Embryonic mice were obtained by caesarean section from pregnant females deeply anaesthetised wth 0.35 ml of 25% urethane in saline (i.p.). Whole embryos (from E7.5 to E10.5) or brains were dissected in ice chilled PBS (from E12.5 to E16.5) (For solutions see appendix 1). Adult brains were obtained after decapitation of anaesthetised animals

and dissection. All tissue was fixed overnight in 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS), followed by rinsing and storage in PBS at 4°C. Where necessary, the extra embryonic membrane or tail tip was removed and stored at -20°C for subsequent genotyping.

2.3 Tissue processing

Brains were embedded in paraffin wax using an automated tissue processor. This was a 17 hour cycle in which the brains were dehydrated through an alcohol series from 50% to 100% ethanol, followed by 4.5 hours in xylene. The brains were sectioned at 10μm in a coronal plane and mounted onto TESPA (3-aminopropyl-triethoxysilane) coated slides. For whole mount *in situ* hybridisation, embryos were dehydrated through a series of methanol and PBS, from 25% methanol to 100% methanol. Whole embryos were then stored at -20 °C until needed.

2.4 Genotyping

2.4.1 DNA extraction

The tails of embryos were removed and digested overnight in 500μl tail-tip-lysis-buffer (TTLB) with Proteinase-K (10mg/ml) at 55°C in a shaking water bath. An equal volume of phenol chloroform was added and mixed for at least ten minutes. The mixture was centrifuged at 13000g for two minutes and the aqueous phase retained. 1ml of EtOH (per 400μl) + 1/10th volume NaOAc (pH5.5) was added to precipitate the DNA which was extracted, dipped in 70% EtOH and transferred to a clean eppendorf. This was air dried

for ten minutes, and re-suspended in 50µl Tris EDTA buffer by shaking overnight at 37°C to give an approximate DNA concentration of 0.5µg/µl.

2.4.2 Southern blot analysis

DNA digestion and electrophoresis

Samples of DNA were digested as follows: per digest: 20µl DNA (from the above digest), 4µl 10XH Buffer (Boehringer), 2µl Spermidine (200mM), 2µl EcoRI (80 units) and 12µl dd$H_2$0 were added into a 1.5ml eppendorf. This mixture was placed at 37°C for five hours and 20µl ran on a 0.8% agarose gel at 40V for 16 hours. The DNA in the gel was then photographed under ultra violet light and subsequently transferred to a nylon positively charged membrane (Roche) as follows; First, the gel was treated with 0.25N HCl for 30 minutes, Denaturing solution for 2X30 minutes, and Neutralisation solution for 2X30 minutes (See Appendix 1, page 200, for solutions). The gel was then placed on a platform inside a tray suspended above a solution of 20XSSC. A nylon membrane was then placed on top of the gel, and on top of this, two pieces of blotting paper saturated with 2XSSC. Air bubbles were smoothed out and absorbent towels placed on top to draw the SSC (and with it the DNA) into the membrane. This was left for 12-24 hours at room temperature. Afterwards, the membrane was air dried and baked for 30 minutes to two hours at 120°C.

Probe production and hybridisation

A 3' DNA probe (a 110 base pair PstI fragment) labelled with ^{32}P was made to the *Wnt8B* allele using a high-prime-DNA labelling kit (Boehringer), following the manufacturers instructions. This probe was added to the membrane and left to hybridise over night at 65°C. After hybridisation, the membrane was washed (three times, for 20 minutes each time) with Church wash solution (See Appendix 1, page 200), wrapped in cling film to retain moisture, and exposed to X-ray film for visualisation of radioactivity.

2.5 *In situ* hybridisation

2.5.1 Production of plasmid DNA

50mls of L-Broth were inoculated from single colonies of bacteria harbouring the plasmid of interest. The bacteria were left to grow at 37°C for 16 hours, shaking at 200 rpm. This culture was then centrifuged at 13000g for 15 minutes and the supernatant disposed of. Plasmid DNA was purified by alkaline lysis using a midiprep kit, following the manufacturers instructions (Qiagen - plasmid midi kit 10).

2.5.2 Probe linearisation

In a 1.5ml eppendorf, 10µg plasmid DNA, 5µl buffer H (Boehringer), 38µl ddH_2O and 4 units of restriction enzyme were added and incubated at 37°C for four hours. The digest were subsequently purified by (1:1) phenol chloroform extraction and ethanol precipitation. The pellet was air dried for ten minutes and re-suspended in RNAse-free ddH_2O.

2.5.3 Probe transcription

The linearised plasmids were transcribed using the Boehringer Mannheim DIG RNA labelling kit. Transcription reactions were carried out in a final volume of 20µl. For both sense and antisense reactions, 2µg linearised plasmid DNA, 2µl 10X digoxygenin NTP labelling mix, and 2µl 10Xtranscription buffer were added into a 1.5ml eppendorf and the volume made up to 18µl with ddH_2O. Two units of the appropriate RNA polymerase was added and the reactants mixed and placed at 37°C for two hours. 2µl of 0.2M EDTA was added and RNA precipitated with 2X volume of 100% EtOH and one tenth volume of 3M NaOAc at -70°C for one hour. After centrifugation at 13000g for fifteen minutes, the supernatant was removed and the pellet washed with 50µl of 70% EtOH, and centrifuged again for five minutes. The supernatant was removed and the pellet air dried for ten minutes, and re-suspended in 100µl T.E. The yield of DIG labelled RNA was estimated after running a sample of the probe on a 0.8% agrarose gel at 30V for one hour.

2.5.4 Whole mount *in situ* hybridisation

Whole-mount *in situ* hybridisation were performed as described below on E7.5 - E10.5 embryos and E12 brains, following the method of Henrique et al (1995).

Pre-hybridisation

Embryos were rehydrated through 75%, 50%, 25% methanol/PBST, PBST. Embryos were then immersed in 10µg/ml proteinase-K in PTW for 5 - 20 minutes, rinsed in PBST

and post fixed for 5 - 15 minutes in 4% paraformaldehyde in PTW. Embryos were washed once in PBST.

Hybridisation

The embryos were rinsed once in 1:1 PBST/hybridisation mix and left to settle. This solution was replaced with 1ml hybridisation mix and incubated for 3 hours at 65°C. This was replaced by 100ng of DIG-labelled probe in 1ml hybridisation mix and incubated overnight at 65°C.

Post hybridisation

Embryos were rinsed twice in pre-warmed (65°C) hybridisation mix, and then washed (2X30 minutes) in the same solution. Following a ten minute wash in 1:1 hybridisation mix/MABT at 65°C, the solution was left to cool and replaced with MABT. This solution was replaced by MABT/ 2% blocking reagent (Boehringer) for 1 hour followed by 1 hour incubation in the above solution + 20% heat-treated serum. Embryos were incubated over night at 4°C in a 1:2000 dilution of anti-DIG-AP antibody (Boehringer) in the above solution.

Colour detection

The antibody solution was removed and embryos were washed 3X1 hour with MABT, before being washed twice in NTMT for ten minutes each. The embryos were then placed in colour detection reaction (10mg/ml NTMT-NBT/BCIP) for 20 minutes to three days at room temperature. When the colour reached the desired intensity, the embryos were fixed in 4% paraformaldehyde/PBST overnight at 4°C. The staining pattern was then photographed.

2.5.5 Wax section *in situ* hybridisation

In situ hybridisation using digoxygenin-labelled probes were performed on wax sections from E12 and E16 embryos.

Pre-hybridisation

Slides were placed in a glass slide dish containing 2XSSPE for 5 minutes. Slides were incubated at 37°C in 20μg/ml of proteinase-K in P-buffer, fixed in 4% paraformaldehyde at room temperature and placed into 0.2M HCl at room temperature. Each of these steps was fifteen minutes and were all followed by a five minute wash in 2XSSPE. Slides were acetylated by immersing in 0.5% acetic anhydride in 0.1M TEA for ten minutes. Slides were placed into 2XSSPE until the probe was applied.

Hybridisation

The appropriate volume of probe was added to hybridisation mix to give a concentration of 80ng per slide (60μl of solution per slide). The probe was heated to 80°C for four minutes, plunged into ice for two minutes, centrifuged briefly and applied to the slides. Slides were cover-slipped and incubated overnight at 65°C in a humidified chamber.

Post-hybridisation

Slides were placed into 2XSSC at 50°C and left until the coverslips fell off. The solution was then replaced with 2XSSC/50% formamide at 65°C for 45 minutes, 4XSSPE at 50°C for five minutes, 4XSSPE containing 20μg RNAseA at 37°C for 30 minutes and 2XSSC/50% formamide at 65°C for 45 minutes. Slides were then placed into pre-warmed 2XSSC at 50°C and left to cool to room temperature. Slides were permeabilised in PBST for ten minutes and blocked in 1% blocking buffer (Boehringer) in PBST for 30

minutes. Anti-DIG-AP FAB fragments was pre-absorbed in 1% blocking buffer/2% sheep serum/PBST at a 1:2000 dilution for four hours before being applied to the slides which were incubated overnight at 4°C.

Colour detection

Slides were washed in PBST for 3X20 minutes before being washed in alkaline phosphatase buffer for 5 minutes. This solution was replaced by in alkaline phosphatase buffer (100mls) containing 150µl NBT/BCIP (Boehringer). Slides were incubated in the dark until colour developed. Slides were then dehydrated through a series of alcohols and mounted in DPX directly from xylene.

2.6 BrdU immunocytochemistry

Sections were incubated for 25 minutes at 37°C in 100mls of 0.1g trypsin in 0.1g $CaCl_2$, rinsed twice in 100mls Tris buffered saline (TBS) containing 0.2g Triton-X (TBST) and then immersed in 1N HCl for eight minutes at 60°C. Sections were rinsed in ddH_2O followed by TBST and incubated in 100mls of blocking solution of TBST containing 20g normal rabbit serum for 40 minutes. This was replaced by primary mouse anti-BrdU (Becton Dickenson; 1:200) for one hour at room temperature. Following three washes in TBST the sections were incubated in biotinylated rabbit anti-mouse Ig antibody (Dako; 1:200) for 30 minutes at room temperature. BrdU labelled cells were revealed using the peroxidase method described previously (Gillies and Price, 1993) and sections were counter-stained in cresyl fast violet.

2.7 Golgi staining technique

Cerebral cortical hemispheres were placed between two on slides with spacers to ensure the tissue was not pressured excessively. These were then placed into a gauze lined container with Golgi solution (see Appendix 1.) for 24 hours. The cortices were then placed into 1:1 Ethanol : acetone for three hours, and then into 100% ethanol for fifteen minutes. After this they were placed into 1:1 Anhydrous diethyl ether : Ethanol for 1 day and then embedded in Celloidin for 1 day. The tissue was then sectioned (80μm) using a sliding microtome and hydrated from 75% ethanol to ddH20. Sections were then placed into mmonia hydroxide for 30 minutes, washed in dH20 for 30 seconds and placed into buffer solution (see Appendix 1.) for 5 minutes before being placed into 0.2% Methyl Blue Chloride (in buffer) for 60 minutes. Sections were placed in Xylene for 1 minute, and then mounted onto slides for photography.

CHAPTER 3. The role of *Wnt8B* in embryogenesis

3.1 Abstract

A novel member of the mouse *Wnt* gene family, *Wnt8B*, was previously identified in this lab, and a knockout mouse line lacking *Wnt8B* was generated (Richardson et al, 1999). *In situ* hybridisation for *Wnt8B* from E7.5 – E16.5 showed that this gene is expressed in a highly restricted pattern in the developing forebrain, corresponding to the region from which the hippocampus and a number of other adult structures are derived.

To investigate a possible role for *Wnt8B* during development, histological staining was used to examine tissue morphology at different developmental stages in wild type and $Wnt8B^{-/-}$ littermates. A number of lines of evidence clearly link *Wnt* genes with the regulation of cell proliferation and morphogenesis. Therefore, a preliminary analysis of cell proliferation at E12 was carried out using 5'-bromodeoxyuridine (BrdU) labelling analysis along the axis of the medial telencephalic wall in one pair of $Wnt8B^{+/+}$ and $Wnt8B^{-/-}$ littermates. Subsequently, BrdU labelling analysis of the medial wall of the telencephalon in three pairs of $Wnt8B^{+/+}$ and $Wnt8B^{-/-}$ littermates was carried out at E14.5.

These analysis revealed that the expression of *Wnt8B* remains largely restricted to the medial telencephalic wall during development. No detectable effects of the loss of this gene on cell proliferation or tissue morphology were detectable at the ages analysed.

3.2 Introduction

Shortly after gastrulation and neurulation, differential rates of cell division in the anterior neural tube produce three swellings which develop into the forebrain (prosencephalon) midbrain (mesencephalon) and hindbrain (rhombencephalon) (see-Kaufman, 1992). As described in chapter 1, these large areas of tissue are further specified and developed under the control of genes specifically expressed to implement a program of brain development (Shimamura and Rubenstein,1997). The mechanisms involved in early development and regional specification of the forebrain remain comparatively unknown. Elucidating the functions of genes expressed in this area during development will enhance such an understanding, and this is being achieved using the techniques of molecular biology, and commonly, generating knockout animals deficient in the expression of one or more genes, so that by comparison to their wild type counterparts their function may be ascertained. Table 1. Describes how a number of Wnt mutants have been made in the mouse, and the resultant phenotypes.

In this study a *Wnt8B* knockout was used in which the last three of five exons of the *Wnt8B* gene were deleted (See figure 3.1). A null mutation of *Wnt8B* was generated by replacing most of the coding sequence of *Wnt8B* with a neomycin resistance cassette (driven by a beta-actin promoter). Genomic 129/Ola clones containing the *Wnt8B* coding sequence were isolated from a bacteriophage lambda library (obtained from Dr A. J. H. Smith, Centre for Genome Research, University of Edinburgh). Restriction endonuclease and partial sequencing analysis were used to identify *Wnt8B* coding sequences. Homology arms were subcloned from the isogenic lambda clone. The 5'

homology arm comprised a 3.5kb fragment that extends from an XbaI site in intron 3 to an SfiI site that was introduced at the end of the lambda clone. The 3' homology arm comprised a 5.1kb fragment extending from the NotI site at the very end of the coding sequence in exon 6 to a HindIII site 5kb downstream of the end of the *Wnt8B* coding sequence. Correctly targeted clones, in which exons 5 and 6 of *Wnt8B* (which encode the C-terminal two thirds of the protein) are replaced by the neomycin resistance cassette were identified by Southern blotting using 5' and 3' flanking probes. Two separate clones were injected into C57Bl/6 blastocysts to generate chimeric mice. Chimeric males from ES clones 9 and 81 successfully transmitted the targeted allele through the germline, generating heterozygous mice which were then intercrossed to yield $Wnt8B^{-/-}$ homozygotes. In contrast to most other *Wnt* null mutants, homozygous *Wnt8B* null animals are viable and fertile (J.Mason, personal communication).

Wnt gene	Nature of mutation	Phenotype
Wnt1 (McMahon & Bradley, 1990)	Insertion in exon-2	Anterior cerebellar defects. Loss of midbrain and some rostral mesencephalon.
Wnt1^{sw} (Thomas et al, 1991)	Single base pair deletion causing premature termination of translation eliminating the carboxy terminal half of Wnt1 including 16 conserved cysteine residues.	Anterior cerebellar defects. Loss of midbrain and some rostral mesencephalon.
Wnt2 (Monkley et al, 1996)	Insertion in exon-2 preventing the production of full length protein.	Placental defects.
Wnt3 (Liu et al, 1999)	Deletion of exon-3 and most of exon-4	Defects in axis formation.
Wnt5A (Yamaguchi et al, 1999)	Deletion of exon-2	Defects in the outgrowth of the face, ears and genitals.
Wnt7A (Parr & McMahon, 1995)	Insertion in exon-2	Patterning defects in the limb.
Wnt7B (Parr et al, 2001)	Insertion in exon-3	Placental defects

Table 3.1

Desription of the nature of the mutation and the resulting phenotype of Wnt knockout mice.

Figure 3.1

A

MFLMKPVCVLLVTCVLHRSHAWSVNNFLMTGPKAYLVYSSSVAAGAQSGIEECKYQFAWDRWNCPERALQL

1 2

SSHGGLRSANRETAFVHAISSAGVMYTLTRNCSLGDFDNCGCDDSRNGQLGGQGWLWGGCSDNVGFGEAIS

3

KQFVDALETGQDARAAMNLHNNEAGRKAVKGTMKRTCKCHGVSGSCTTQTCWLQLPEFREVGAHLKEKYHA

4

ALKVDLLQGAGNSAAGRGAIADTFRSISTRELVHLEDSPDYCLENKTLGLLGTEGRECLRRGRALGRWERR

5 6

SCRRLCGDCGLAVEERRAETVSSCNCKFHWCCAVRCEQCRRRVTKYFCSRAERPPRGAAHKPGKNS

B

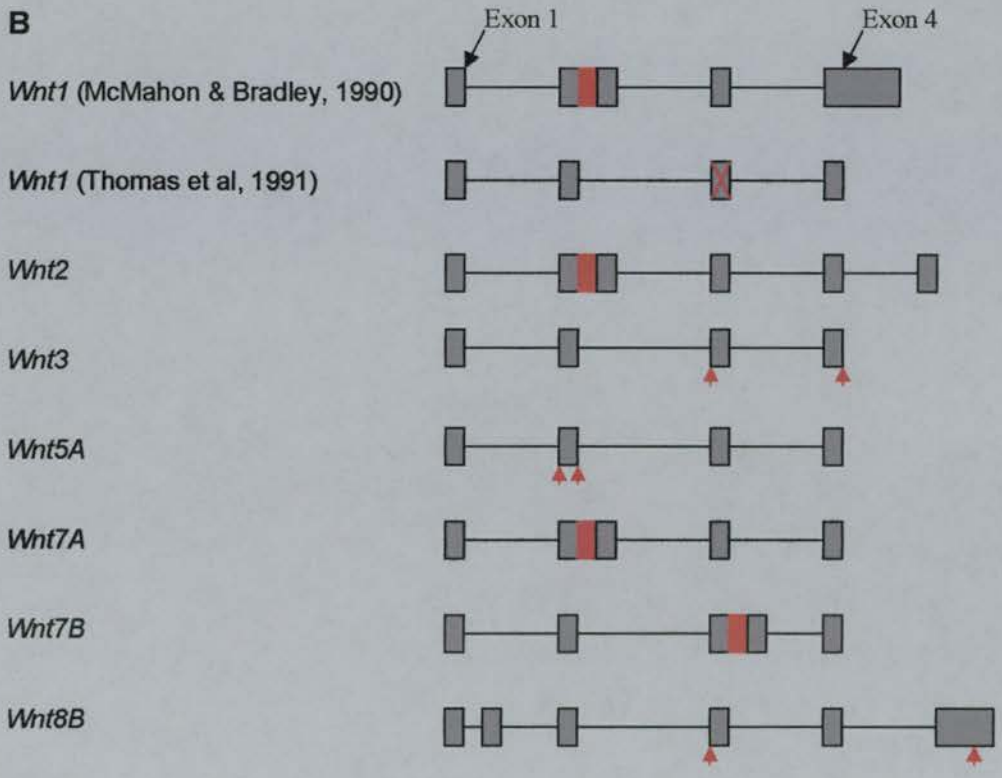


Figure 3.1 The *Wnt8B* translated amino acid sequence and diagram showing how various *Wnt* mutants have been produced. (A) The amino acid sequence of the six exons of *Wnt8B* showing the positions of conserved cystiene residues in red. (B) Representation of the gene structure of *Wnt* mutants showing the number of exons (dark boxes) and the nature of the mutation. A red box bordered by two dark boxes denotes an insertion into a single exon thereby altering the coding sequence so that the correct amino acids are not produced. A red X denotes the deletion of a single base pair, and positions lying between two arrows denote a deleted region. The diagram illustrates the deletion of exons four, five and approximately one half of exon six from the *Wnt8B* gene which, from the amino acid sequence can be seen to contain a number of conserved cystine residues. The *Wnt8B* mutation is comparable to mutations of *Wnt3* and *Wnt7B* which have produced definite null alleles.

Wnt genes are highly evolutionarily conserved and encode a large family of secreted glycoproteins of between 350 and 380 amino acids in length which contain a characteristicly spaced pattern of cysteine residues. *Wnt8B* contains 6 exons and in the mouse is most closely related to the gene *Wnt8A*. The *Wnt* gene family is involved in regulating many developmental processes from as early as the blastula stage (reviewed by Cadigan & Nusse, 1997). Clearly, *Wnt* genes have multiple roles in many systems, although, many are linked with the regulation of cell proliferation and morphogenesis. For example, many *Wnt* genes are expressed in areas undergoing high rates of mitogenesis, and their expression is subsequently down-regulated as mitogenesis is down-regulated (Parr et al, 1993). *Wnt1*, the canonical member of the *Wnt* gene family, when ectopically expressed in the CNS and mammary gland, has been found to dramatically increase the number of cells undergoing mitosis in the ventricular zone of the neural tube, and to produce high levels of adenocarcinomas (Dickinson et al, 1994; Tsukamoto et al, 1988). Additionally, mice lacking both *Wnt1* and *Wnt3A* exibit a reduction in the numbers of dorsolateral neural precursors in the neural tube (Ikeya et al, 1997), and cyclin-D1, a factor controlling the length of the cell cycle has been found to be activated by ß-catenin (and hence, Wnt signalling) acting via the TCF family of transcription factors (Tetsu & McCormick, 1999). These data showing the stimulation of proliferation by some *Wnt* genes, have led to the suggestion that this effect may represent a general model for Wnt signalling in the CNS (Ikeya et al, 1997).

The medial telencephalic wall is formed by an infolding of the superior bridge at around E10 in the mouse, just after closure of the anterior neural tube at this point (see Figure

1.1). Initially it comprises a single continuous sheet of neuroepithelium, areas of which are presumptive for the dorso-medial neocortex, hippocampus and choroid plexus. Additionally, the medial lip of this tissue forms the target, and may provide the guidance mechanisms, for projecting axons from neurons in the frontal cortex, which at the time of contact (E14) are still undergoing active migration to their final destination in layer 5 of the contralateral cortex (Auladell et al, 1995). These axons subsequently form the corpus callosum which provides inter-hemispheric communication, and forms the second major cortical output after the corticothalamic projection. Interestingly, recent work has suggested a role for Wnts in axon remodling and synaptogenesis (Hall et al, 2000;).

As described in Chapter 1, the rapid growth of the telencephalon is primarily due to the extensive proliferation of cells within the ventricular zone, and their migration to the cortical plate. Although little is known about the mechanisms by which the telencephalon becomes regionalised, previous work has suggested that highly localised differences in rates of proliferation may contribute to this process (Polleux et al, 1997). The complex and dynamic nature of the dorso-ventral and rostro-caudal morphology of this area requires a high degree of regulation of localised proliferation, though other processes such as apoptosis, cytoskeletal regulation and cell adhesion are also likely to be crucial. Recently, the expression of members of three large gene families, the *Wnt*s, *BMP*s and *Msx* genes, well characterised as performing important roles in other areas of the brain during early development, have been found to occur in specific restricted and corresponding temporal domains along the axis of the medial telencephalic wall (Furuta et al, 1997; Grove et al, 1998).

3.3 Aims and methods

Aims

i) To determine the detailed expression pattern of *Wnt8B* during early development

ii) To determine any effects of loss of *Wnt8B* on morphology

iii) To determine any effects of loss of *Wnt8B* on proliferation

Methods (for detailed experimental methods see Chapter 2.)

In situ hybridisation was done on whole mount embryos from E7.5 to E12.5 and on coronal wax sections of the medial wall from E12.5 to E16.5, to investigate the expression pattern of *Wnt8B* during early development, and also for use as a guide for BrdU labelling analysis, such that the area chosen for cell counting corresponded the domain of *Wnt8B* expression and to extended beyond it. To look for any histological abnormalities in $Wnt8B^{-/-}$ animals, brains of E12 and E14 embryos were sectioned and counterstained with nuclear fast red dye.

To test the hypothesis that *Wnt8B* contributes to cell proliferation in the medial telencephalic wall, BrdU was injected (i.p. in saline solution 50μg g^{-1}) into time mated mothers in order to label cells in S phase. $Wnt8B^{+/+}$ and $Wnt8B^{-/-}$ littermates at E12 and E14 were subsequently sacrificed one hour post injection, and the brains processed for BrdU detection. Labelling indices at precise points along the dorso-ventral and rostro-caudal axis were determined as a comparative assay for mitogenesis. E12 was chosen for preliminary analysis using one pair of littermates. Subsequently, three pairs were

analysed at E14. These ages were chosen because at this time the medial telencephalic wall is composed solely of a rapidly proliferating ventricular layer, and the morphology is still simple enough to allow an accurate, reproducible and thorough analysis of cell proliferation. Additionally, the expression of *Wnt8B* in this region is very obvious at this time.

Wnt8B Homozygous mutant and wild type embryos were obtained from three separate inbred laboratory colonies which were of different background strains: C57Bl/6, CBA and 129Sv. Heterozygous mice were mated overnight and the following day was deemed embryonic day 1 (E1).

3.4 Results

3.4.1 *Wnt8B* expression during early development

To detect transcripts of *Wnt8B* and other developmentally important genes in whole tissue and on sections, *in situ* hybridisation (ISH) was used. Whole mount *in situ* hybridisation shows that *Wnt8B* is expressed in a restricted domain in the anterior neural tube. As the tissue in this position at this time subsequently joins together medially and then involutes at the midline to form the medial telencephalic wall, it is likely that the domain of expression observed in Figure 3A is equivelent to that in Figure 3B and C. At E7.5, two days before closure of the neural tube commences, *Wnt8B* is strongly expressed on the lateral most tips of the anterior neural tube. This domain of expression is maintained at E8.5 and E10, and the extent of expression appears to expand in conjunction with the growth of this tissue. At E12.5, expression of *Wnt8B* can be seen to extend along the anterior-posterior axis to cover almost the entire length of the medial telencephalic wall from approximately 100μm from the anterior ventricular edge to the posterior ventricular edge (Figure 3.3). In wax sections at E12 showing the dorso-ventral axis, this expression is seen to start at the junction between the epithelium of the choroid plexus and to extend dorsally, ending before it reaches the dorsal epithelium. A gradient of expression is also clearly apparent from the medial to dorsal aspect (Figure 3.4). Matched sections at E12, processed by ISH, for *BMP4*, *Wnt2B* and *Msx2*, show these genes to be expressed in similar domains and gradients (Figure 3.4). Expression of *Wnt2B* and *Msx2* appears to have a similar area of expression, and also to occur in a subset of cells expressing *Wnt8B*. At E14 and E16, coronal wax sections reveal a high

level of expression of *Wnt8B* which is maintained in the medial telencephalic wall in a gradient which is highest ventrally (Figure 3.5).

Additionally, a second area of *Wnt8B* expression corresponding to the hypothalamus is visible from E10 (Figure 3.2d). By E16 this domain of expression becomes localised to the ependymal cells lining the 3rd ventricle along the hypothalamus (Figure 3.6).

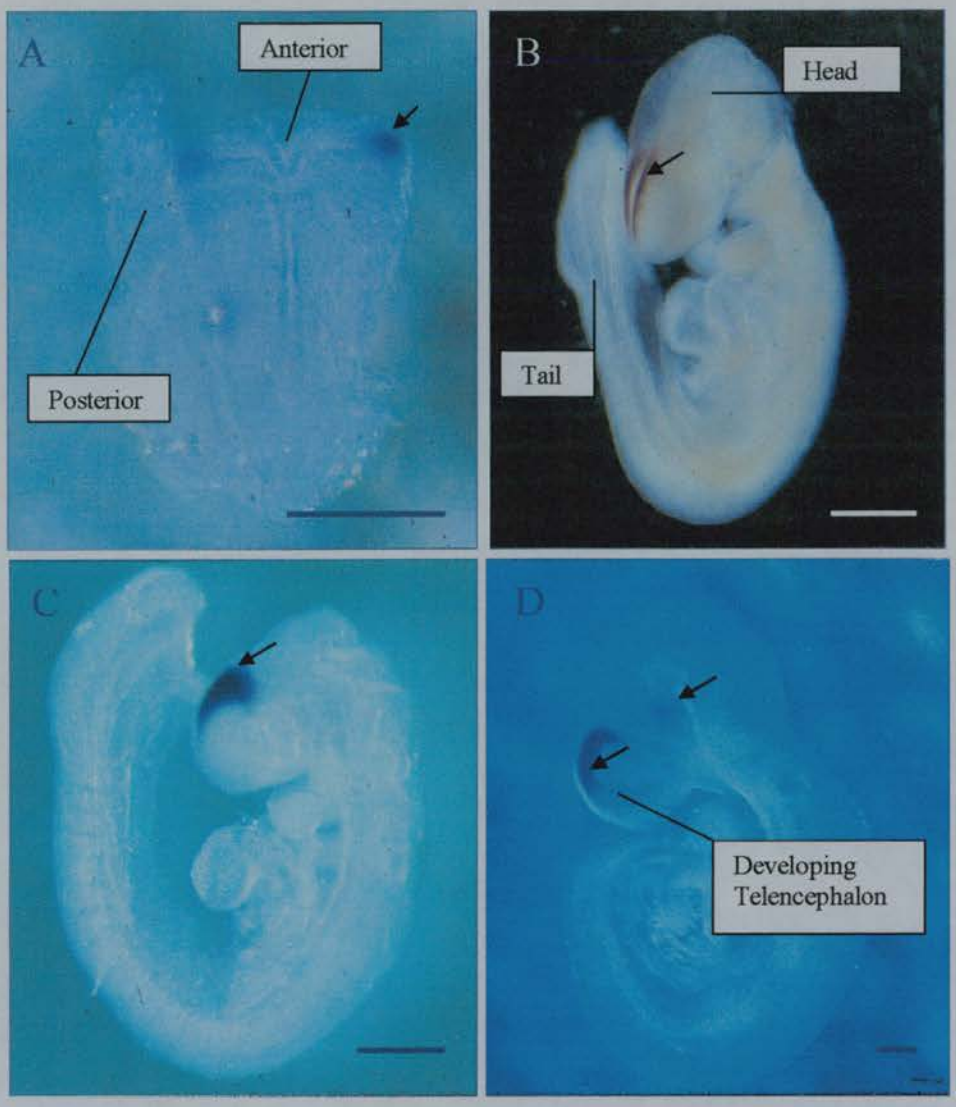


Figure 3.2 Photomicrographs of whole mount *in situ* hybridisation for *Wnt8B* in embryos at E7.5 (A), E8.25 (B) (work done by J Mason), E8.5 (C) and E10 (D). A purple colour denotes the position of expression in the tissue (arrows). (Hypothalamic expression is obscured by the thickness of tissue) Embryos are positioned laterally. Scale bars 0.5mm.

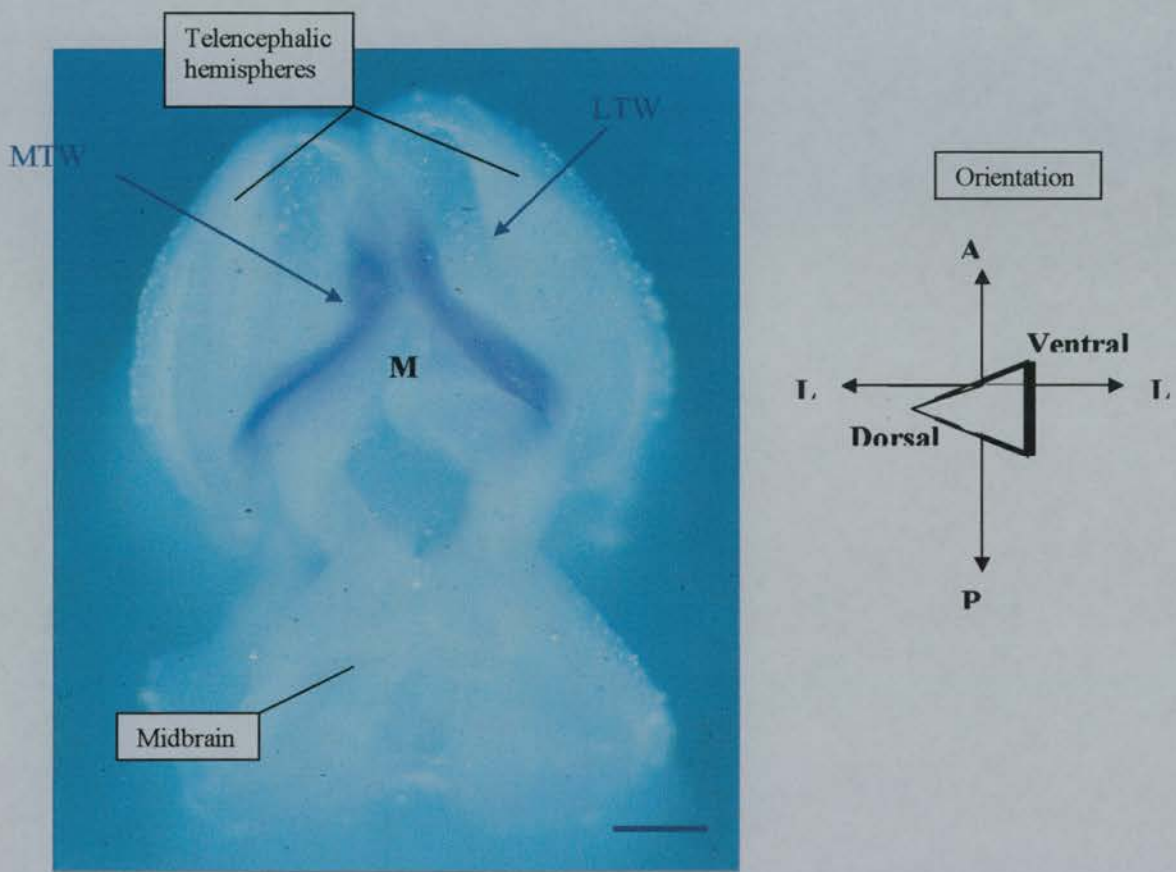


Figure 3.3 Photomicrograph of whole-mount *in situ* hybridisation for *Wnt8B* in an intact brain (complete fore and midbrain) which was dissected from the skull of an E12 embryo. A purple colour denotes the position of expression in the tissue. The brain is positioned with its ventral edge facing the viewer to give a view of expression along the medial telencephalic wall (see orientation bars). Abbreviations: MTW, medial telencephalic wall; LTW, lateral telencephalic wall. A, anterior; P, posterior; L, lateral; M, medial plane. Scale bars 0.5mm.

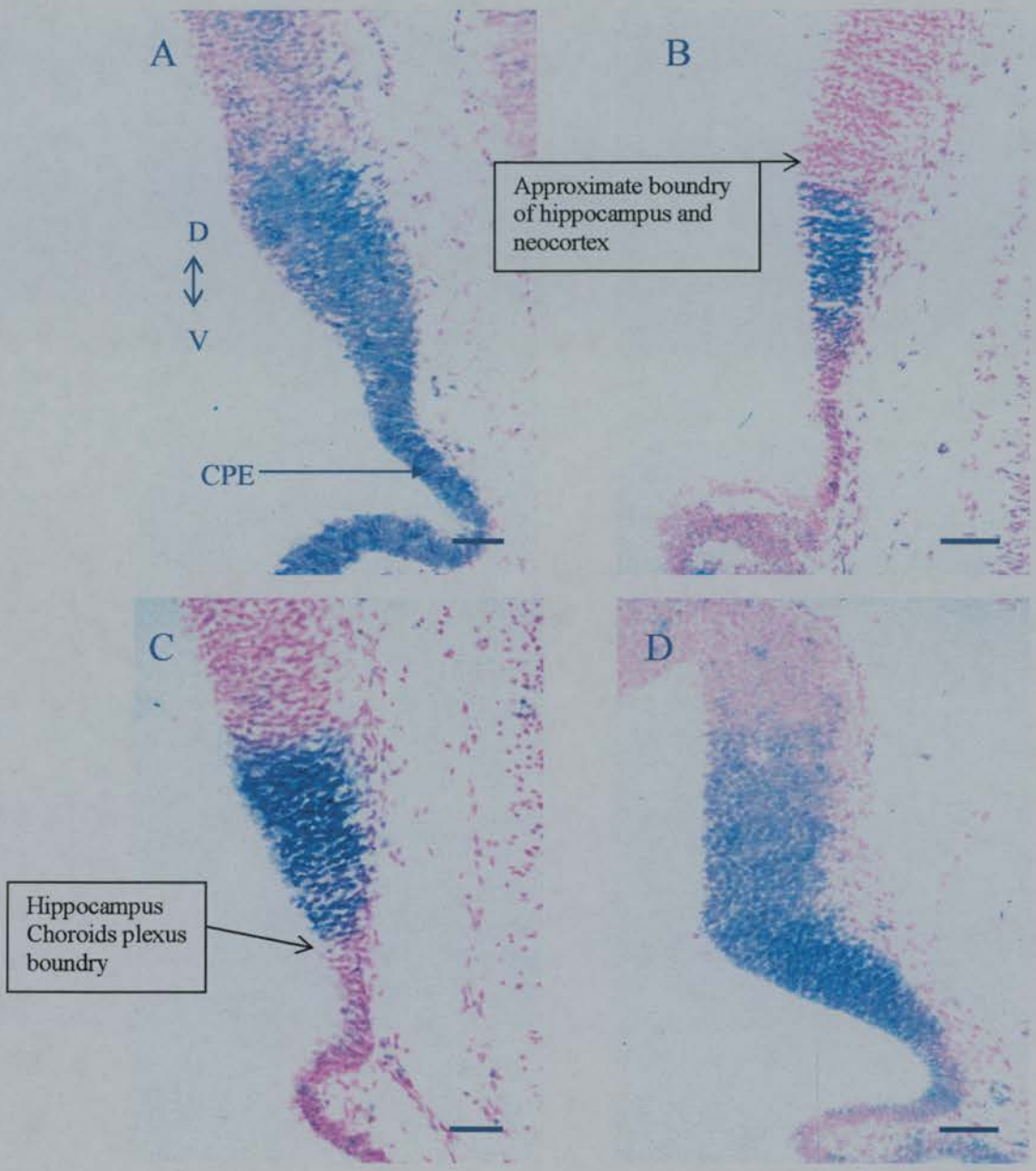


Figure 3.4 *In situ* hybridization in coronal wax sections of the medial telencephalic wall at E12. *In situ* hybridization for *BMP4, Msx2, Wnt2B & Wnt8B* (A-D respectively), illustrating restricted domains of expression. Sections are counterstained with nuclear fast red dye. Abbreviations: CPE, choroid plexus epithelium; D, dorsal; V, ventral. Scale bar 50µm.

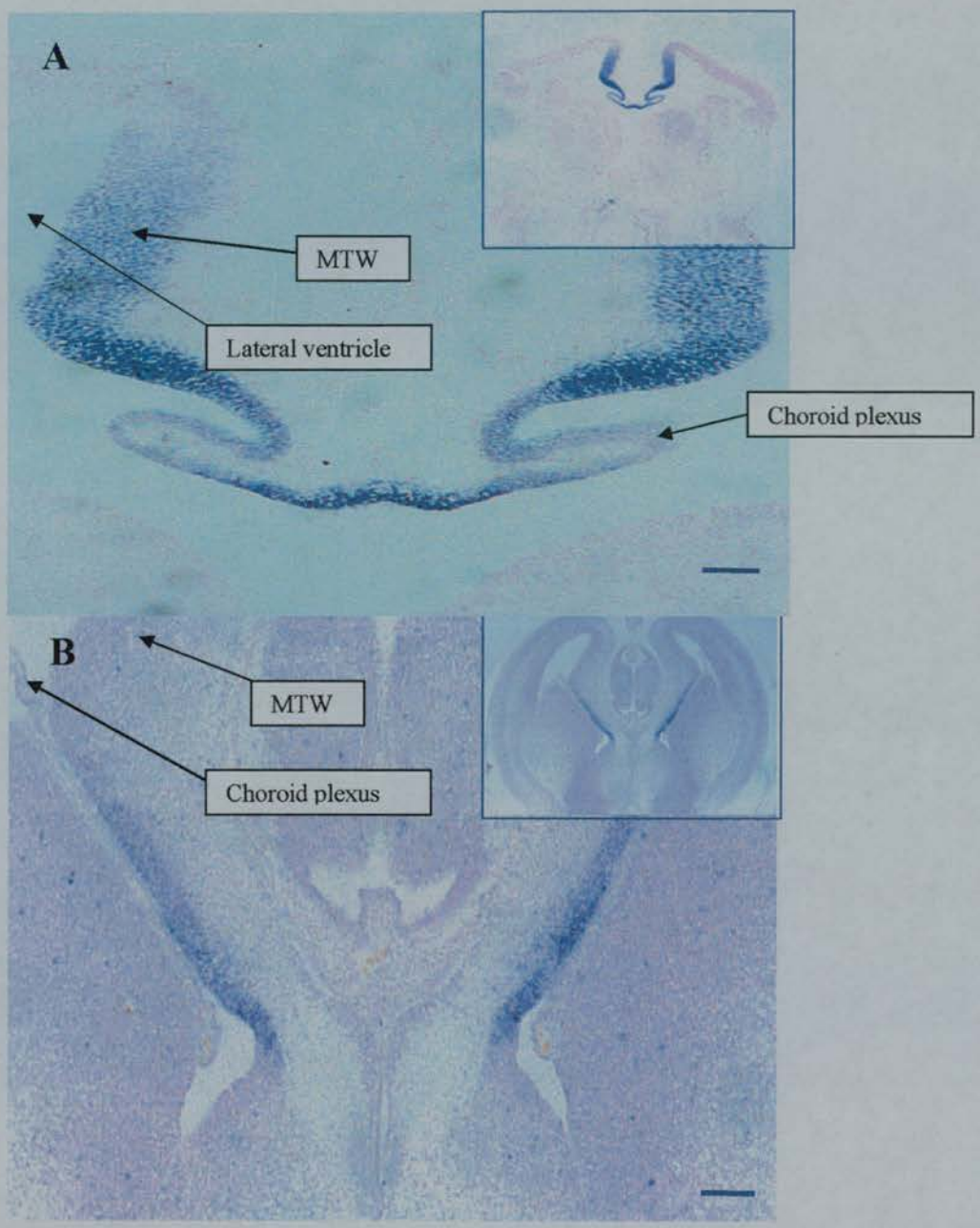


Figure 3.5 ***In situ*** hybridization for *Wnt8B* in coronal wax (the level from which the sections were taken is indicated in Figure 3.6 by a dashed white line). Sections of the medial telencephalic wall at E14 (A) and E16 (B). Insets show low power micrographs of the complete sections. Scale bar 50μm. Abbreviations: MTW, medial telencephalic wall. Scale bar 50μm

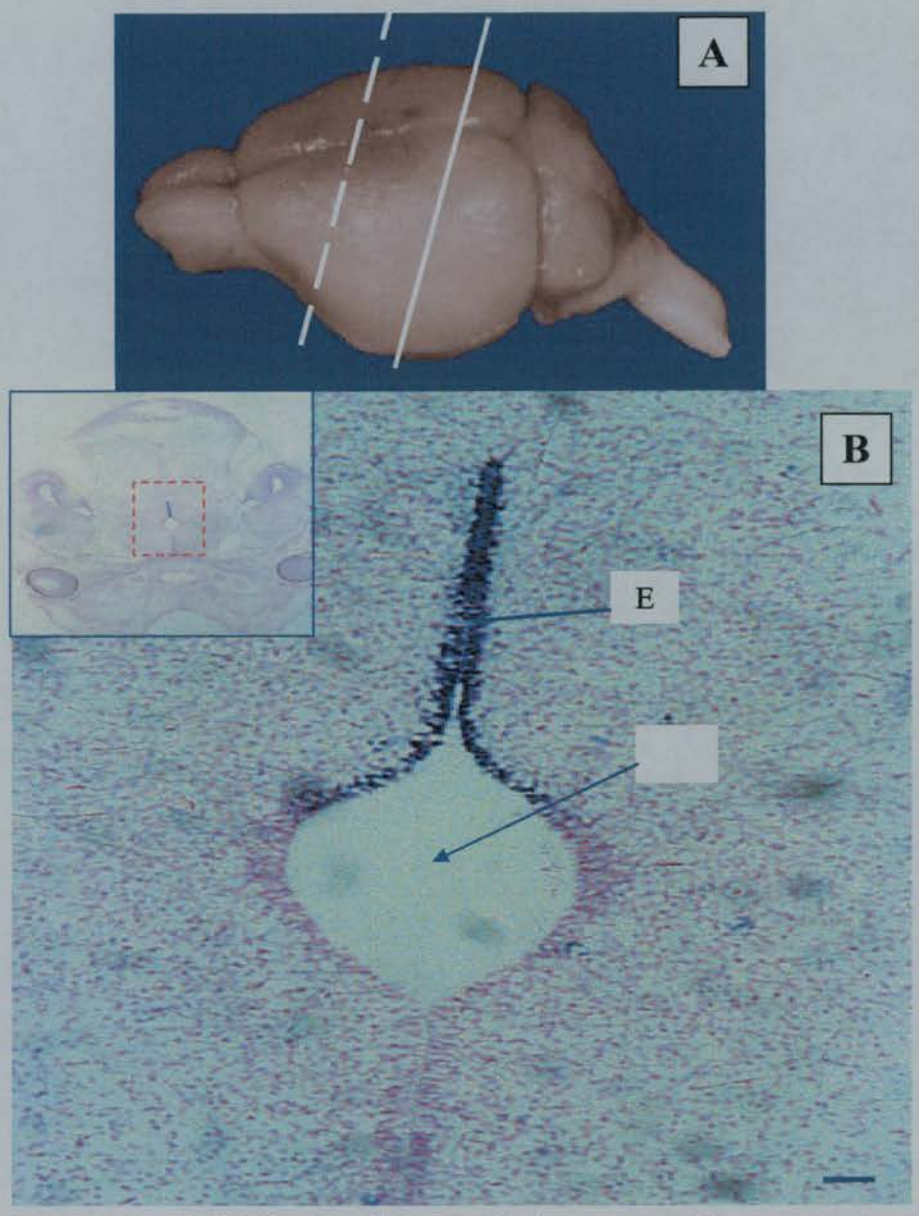


Figure 3.6 *In situ* hybridization for *Wnt8B* in a coronal wax section of the hypothalamus at E14, indicating expression in the ependymal cells of the hypothalamus. Whole mouse brain indicating with a solid white line the level from which the section was taken. Picture from www.brainmuseum.org (A). *In situ* hybridization for *Wnt8B* (B). Insets show low power micrograph of the complete section. Abbreviations: V3, third ventricle; E, ependymal cell layer. Scale bar 50µm.

3.4.2 Histological examination of *Wnt8B* null mice

Evidence for morphological and histological abnormalities were investigated in *Wnt8B*$^{-/-}$ embryos from E12.5 to E16.5. To do this, coronal sections of brains from E12.5 and E16.5 *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ littermates were counter-stained with nuclear fast red dye to reveal cellular detail, and examined using light microscopy. *Wnt8B* expression was found in the anterior dorsal neural tube and hypothalamus at E12.5 and E16.5 (Figures 3.3, 3.6). Therefore, attention was given to these structures. However all areas of the brain were examined since Wnt8B may constitute a diffusible factor and as such may exert its effects at a distance from its site of expression. In the embryonic stages analysed, attention had to be paid to the rapid appearance and development of many structures such as the hippocampus, choroid plexus, expansion of the neocortical wall, thalamus and hypothalamus, and cortical lamination. Especially regarding tissue thickness, it was necessary to examine multiple animals because the difficulty in sectioning all tissue at an equivalent angle can cause the appearance of some structures to be altered. This was misleading at E14, as it appeared from one pair of *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ littermates that the epithelium of the medial telencephalic wall was thinned in the *Wnt8B*$^{-/-}$ embryo, thereby agreeing with the hypothesis for a possible role of *Wnt8B* in proliferation. Analysis of a larger number of animals revealed this to be the result of different angles of tissue sectioning (data not shown).

Previous studies have found that the phenotype displayed by a particular mutation can vary depending upon the genetic background on which it is expressed. For example, apparent null mutations at the *Wnt1* locus produce phenotypes ranging from mild

cerebellar defects causing ataxia to complete deletion of midbrain and some hindbrain leading to neonatal lethality (Thomas & Capecchi, 1990; McMahon & Bradley, 1990). On careful analysis of E12 and E14 *Wnt8B*$^{-/-}$ embryos from c57/Bl6, CBA and 129/ola backgrounds, no gross or subtle change in histology, or in the timing of the growth of structures could be distinguished in *Wnt8B*$^{-/-}$ from their *Wnt8B*$^{+/+}$ littermates (data not shown).

3.4.3 Preliminary BrdU analysis at E12

Analysis

To determine whether proliferative rates in the medial telencephalic wall of *Wnt8B*$^{-/-}$ embryos were altered, one pair of E12 *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ littermates were injected with BrdU and sacrificed after one hour. Brains were serially sectioned (coronally at 10μm) and from sections comprising the cortex, every fifth section was taken for BrdU immunocytochemistry (see Figure 3.7). Camera lucida drawings were made of the dorso-ventral wall of the telencephalon in each of these sections as indicated in Figure 3.8. A reference point was chosen at the anatomical junction between choroid plexus and cortical epithelium at which point there is a marked thinning of the epithelium (at positions rostral and caudal to the choroid plexus, an equivalent point of reference was chosen). Figure 3.8 illustrates the how the medial telencephalic wall was divided into 100μm bins for cell counting. The numbers of BrdU labelled and unlabelled cells from the same bin (of the dorso-ventral aspect of the medial telencephalic wall), from every fifth section through the anterior-posterior axis of the cortex, were used to calculate the

labelling indices (LI: labelled cells as a proportion of total cells; Takahashi et al, 1993) for that position. These labelling indices were plotted against distance dorso-ventrally to give a complete profile of proliferation along this axis of the cortex (Figure 3.9a).

Labelling indices from the medial wall of the telencephalon were also plotted against distance along the anterior-posterior axis of the cortex. These data were derived from the numbers of BrdU labelled and unlabelled cells counted in the medial wall from each of the sections along the anterior-posterior axis (Figure 3.9b).

The total number of BrdU labelled cells counted was also expressed as a percentage of the total numbers of cells counted in *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ embryos and plotted as a bar graph (Figure 3.9c). From *in situ* hybridisation for *Wnt8B* at E12 and E14 it was known that the area of cell counting comprised epithelium both expressing and not expressing *Wnt8B* (see Figure 3.10)

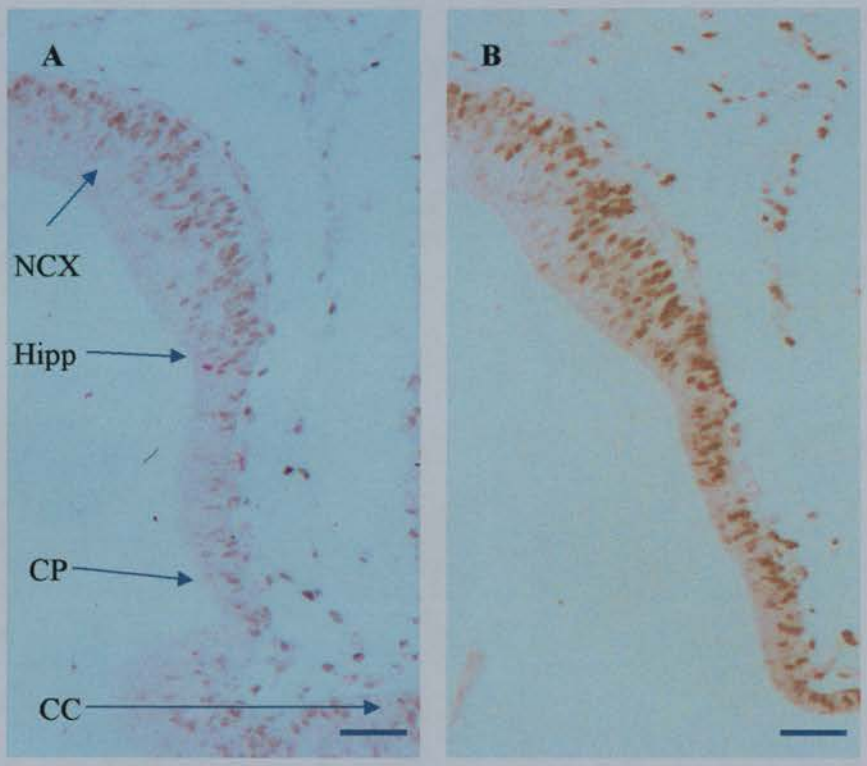


Figure 3.7 Photomicrographs of E12 coronal sections of the medial telencephalic wall in *Wnt8B*$^{+/+}$ (A) and *Wnt8B*$^{-/-}$ (B) embryos. BrdU labelled cells are denoted by a dark brown colour, and the tissue is counter-stained with nuclear fast red dye. The structures which develop from this region of epithelium are indicated. Abbreviations: CP, choroid plexus; NCX, neocortex; Hipp, hippocampus; CC, corpus callosum. Scale bar, 50μm

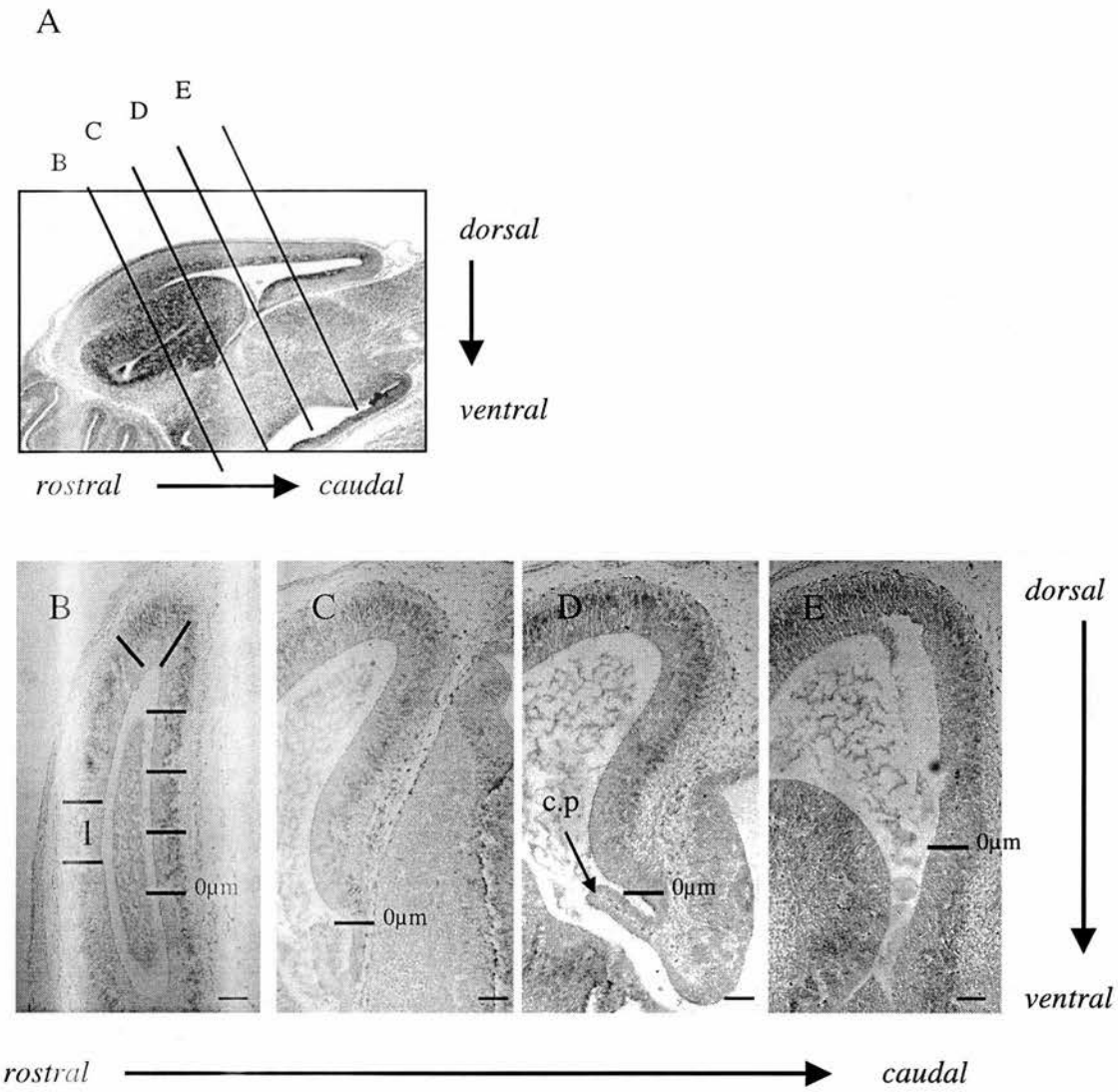


Figure 3.8 Representation of BrdU labelled E12 coronal sections at different positions through the rostro-caudal axis of the medial telencephalic wall indicating the position of the reference point from where the dorso-ventral epithelium was divided into 100µm bins for BrdU quantification. Abbreviations: cp, choroid plexus; l, lateral wall of telencephalon . Scale bars, 50µm

Proliferation along the dorso-ventral axis of the telencephalon

In the *Wnt8B*$^{+/+}$ embryo, labelling indices plotted against distance dorso-ventrally from the point of reference are stable for a distance of 100μm and then rise by approximately 30% over the next 200μm. A further rise of approximately 10% then occurs over the next 200μm (see Figure 3.9a). These changes in proliferation seem to coincide with the relative differences in thickness which occur in the epithelium of the medial telencephalic wall at E12. Hence, from the reference point, the tissue thickness remains relatively constant for 100μm at around seven cell diameters from ventricular to pial edge, before a rapid increase in thickness occurs over the next 200μm, which more gradually increases towards the dorsal most aspect of the epithelium. On looking at the numbers of BrdU labelled cells in vivo relative to the thickness of tissue in which they lie, this relationship is striking and it is strongly indicative of the process of radial migration predominating at this age. Labelling indices obtained for the *Wnt8B*$^{-/-}$ embryo indicate that there is no deviation in proliferative rates, away from that of the wild type littermate at any point along the dorso-ventral axis of the medial telencephalic wall.

Proliferation along the rostro-caudal axis of the telencephalon

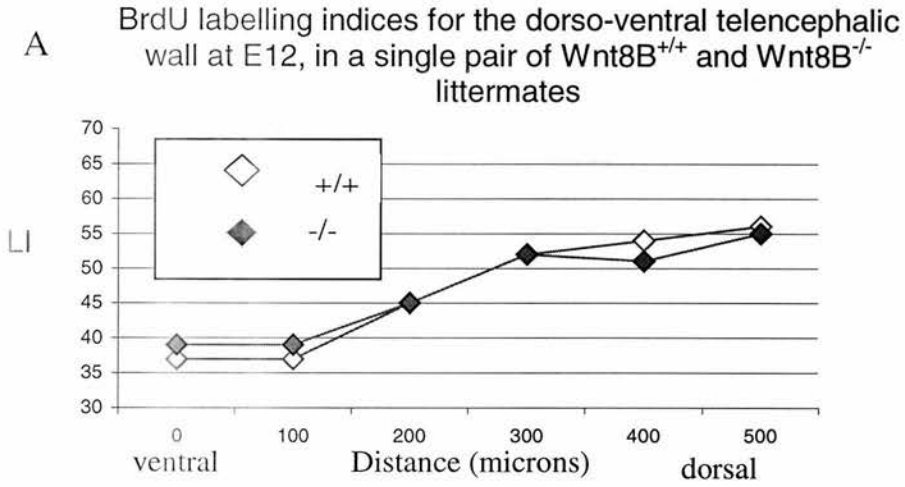
In the *Wnt8B*$^{+/+}$ embryo, labelling indices plotted against distance rostro-caudally indicate that proliferative rates decline by approximately 50% from the anterior to the posterior edges of the medial telencephalic wall. Additionally, this decrease appears neither to occur gradually, nor indeed at one point, but over a number of sinusoidal cycles which seem more erratic than the profile of proliferative rates in the dorso-ventral

axis (see Figure 3.9b). The more erratic profile compared to that seen along the dorso-ventral axis is likely to reflect the greater morphological differences which occur along the rostro-caudal axis. Labelling indices obtained for the *Wnt8B*$^{-/-}$ embryo along the rostro-caudal axis indicate that, as in the dorso-ventral axis, there is no deviation in proliferative rates, away from that of the wild type littermate. In both the proliferative gradient and fluctuations along the rostro-caudal axis, the labelling indices overlap or closely follow one another.

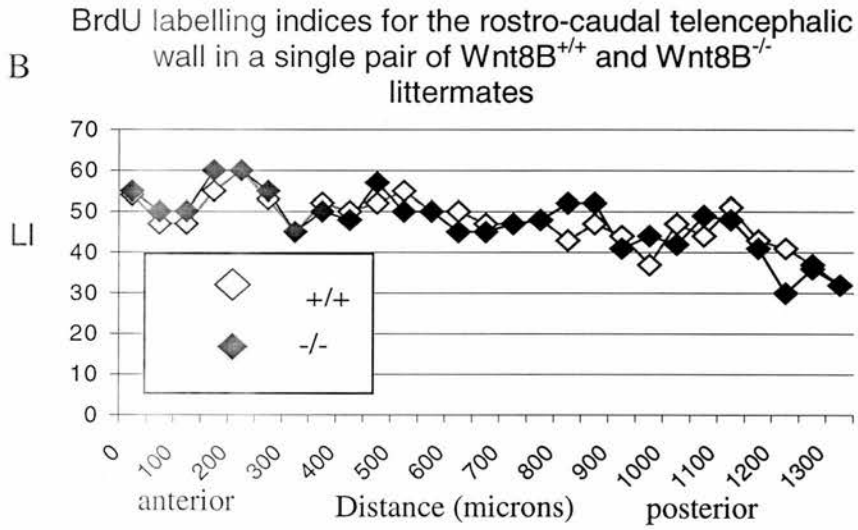
Figure 3.9c illustrates the overall percentage of BrdU labelling at E12 in both *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ littermates. A 1.7% difference exists between the two embryos with the slightly lower figure representing the wild type. However as only one pair of mice were used no statistical significance can be drawn from the results.

Figure 3.9

A



B



C

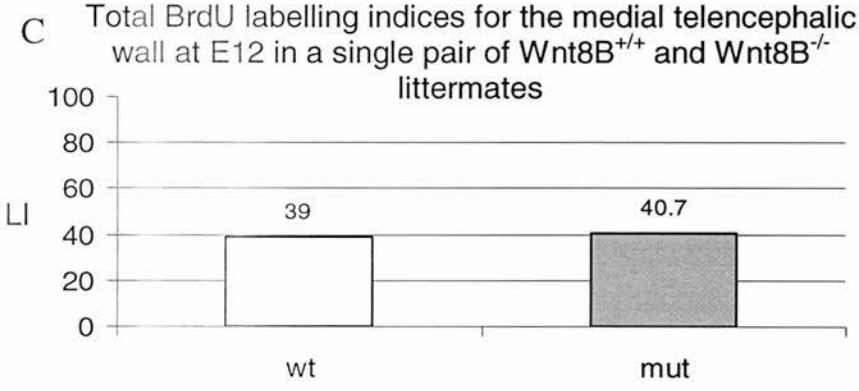


Figure 3.9 BrdU labelling indices in the dorso-medial wall of the telencephalon at E12 in one pair of *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ littermates. (A) Graph illustrating the BrdU labelling indices at each 100 micron bin in the medial wall of the telencephalon, from the ventral reference point to the dorsal most aspect of the wall at E12 in a *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ littermate (numbers of labelled and unlabelled cells from the same position of the dorso-ventral aspect of the medial telencephalic wall, from every fifth section through the anterior-posterior axis of the cortex, were used to generate the labelling indices for that position). (B) Graph illustrating BrdU labelling indices in the medial wall of the telencephalon every fifty microns from the anterior to posterior edges of the cortex at E12 in a *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ littermate (from coronal sections taken every fifty microns along the anterior-posterior axis of the cortex, numbers of labelled and unlabelled cells in the medial wall of the telencephalon from each section were used to generate labelling indices). *Wnt8B*$^{+/+}$ data denoted by open diamonds. (C) Overall BrdU labelling indices for one pair of *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ embryos at E12. *Wnt8B*$^{+/+}$ data denoted by un-shaded box. Abbreviation: LI, labelling indices.

3.4.4 BrdU analysis at E14

Analysis

Histological analysis of one pair of *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ embryos at E14 suggested that the medial telencephalic wall was thinner in the *Wnt8B*$^{-/-}$ embryo, hence a larger number of animals (three pairs of E14 *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ littermates) was used to undertake a more thorough analysis at this time point in development. Sections approximately 25% (rostral), 50% (mid) and 75% (caudal) from the anterior most tip of the cortex (ie. The total cortex was sectioned and the number of sections was used to determin the percentage depth from the anterior edge) were taken for BrdU analysis. Figure 3.10 illustrates how the medial telencephalic wall was divided into 100μm bins for cell counting. Labelling indices were obtained as described in section 3.4.3, with the exception that the mean labelling indices from three Wnt8B$^{+/+}$ and three Wnt8B$^{-/-}$ animals were plotted (not one wild type and one mutant animal as at E12) with the standard errors. Additionally, from each animal, the numbers of labelled and unlabelled cells from corresponding bins, from three serial sections at each level (rostral, mid point and caudal) were combined to generate labelling indices for Wnt8B$^{+/+}$ and Wnt8B$^{-/-}$ animals (whereas at E12 the numbers of labelled and unlabelled cells from corresponding bins, from every fifth section were combined to generate labelling indices). Labelling indices were plotted against dorso-ventral distance only (as serial sections at rostro-caudal points were not analysed at E14). Means were compared statistically using Student's t-test and cell counting was done blind.

Proliferation in the dorso-medial wall of the telencephalon at E14

In the *Wnt8B*$^{+/+}$ embryos, labelling indices at the rostral point indicate a gradual decrease and then increase in proliferative rates along the medial to dorsal extent of the tissue (see Figure 3.11a), and similarly to E12, this may be accounted for by the relative changes in tissue morphology in the medial telencephalic wall (see Figure 3.10a). Data obtained for the *Wnt8B*$^{-/-}$ littermates indicate a less obvious pattern as labelling indices appear more constant through more points in the medial wall. At each point analysed the standard errors of the means of the two samples were overlapping indicating that no significant differences in proliferative rates occur between *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ embryos in the rostral medial telencephalic wall.

In the *Wnt8B*$^{+/+}$ embryos, labelling indices along the dorso-ventral axis at the mid point of the medial telencephalic wall similarly indicated no differences in proliferative rates between *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ embryos (Figure 3.11b). Similarly, labelling indices obtained for *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ embryos in the caudal point indicate no proliferative differences at any point analysed along the dorso-medial wall (Figure 3.11c).

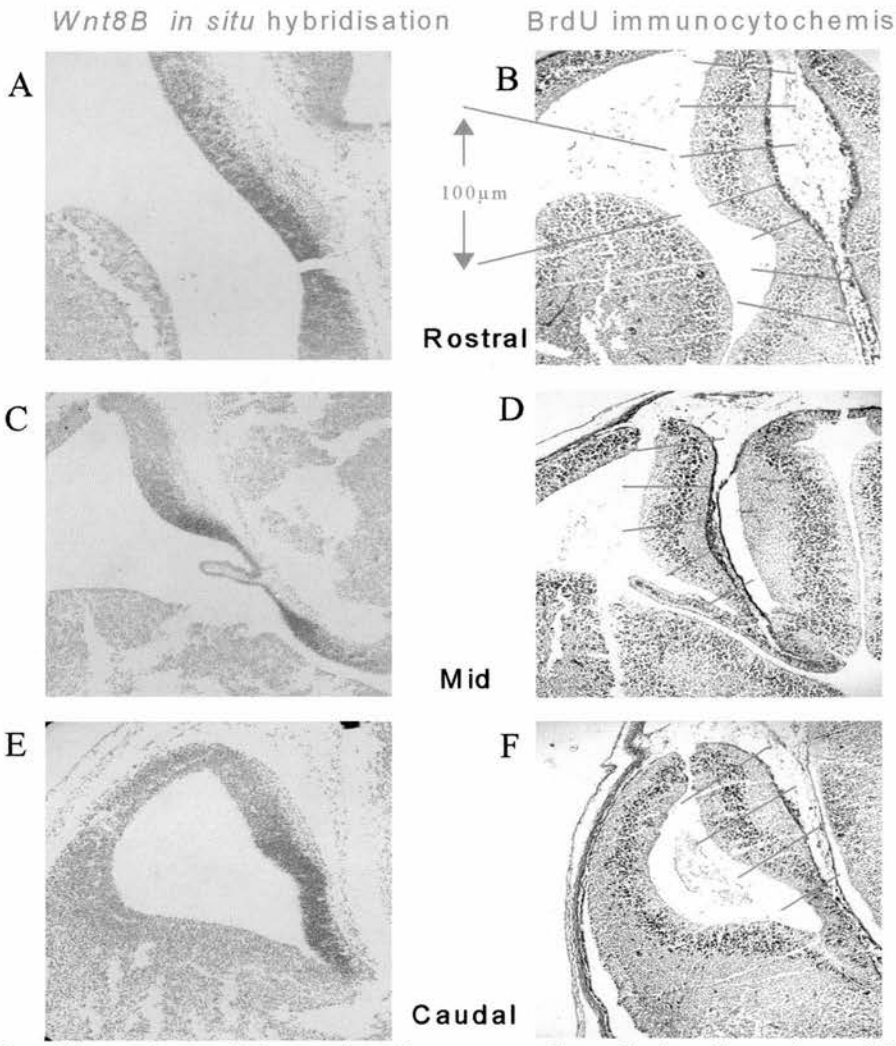
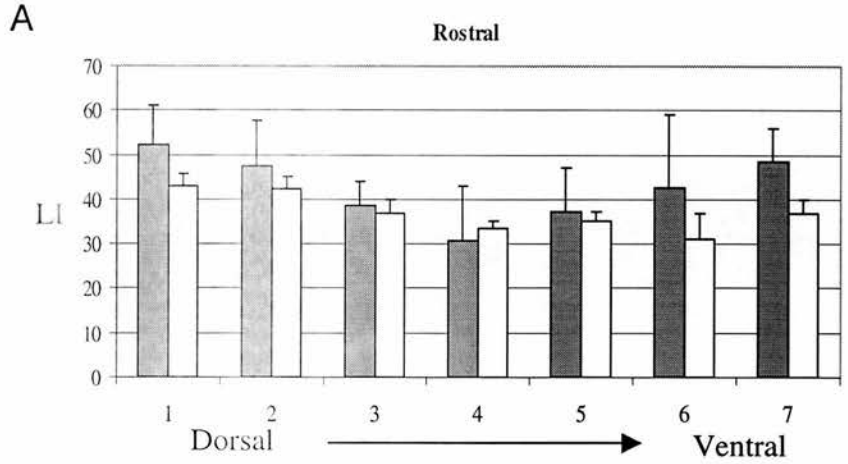


Figure 3.10 Illustration of how the dorso-ventral wall (sections from W*nt8B*$^{+/+}$ shown) of the telencephalon was divided into bins for cell counting at three points along the anterior-posterior axis of the cortex. Photomicrographs of *Wnt8B in situ* hybridisation in E14 coronal sections of the medial telencephalic wall from 25% (rostral)(A), 50% (mid)(C) and 75% (caudal)(E) depth from the anterior cortex (ie. The total cortex was sectioned, and the number of sections was used to determin the percentage depth from the anterior edge). Matched sections of BrdU labelling (B,D and F respectively) indicate the position of the reference point from where the dorso-ventral epithelium was divided into 100µm bins for BrdU quantification.

A


Rostral
70
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LI
1
2
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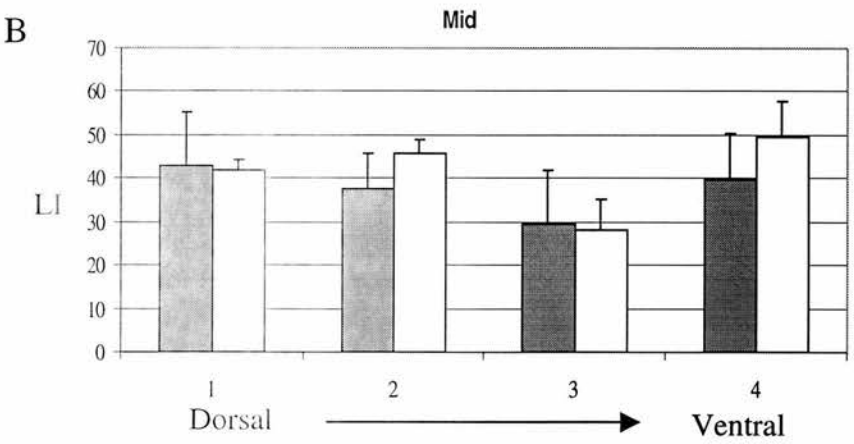
Dorsal
Ventral

B


Mid
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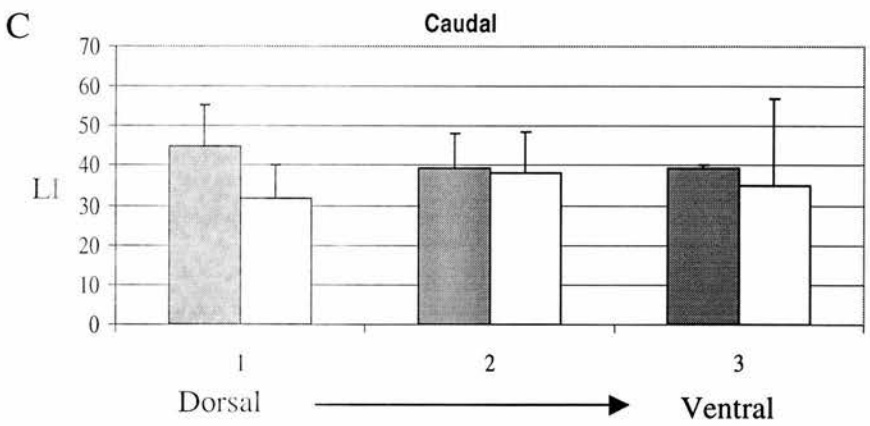
LI
1
2
3
4
Dorsal
Ventral

C


Caudal
70
60
50
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30
20
10
0
LI
1
2
3
Dorsal
Ventral

Figure 3.11 BrdU labelling indices in of the medial telencephalic wall at E14 in three *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ littermates. BrdU labelling indices in the medial wall of the telencephalon are shown at a rostral position (A), a midpoint (B), and a caudal position (C), along the anterior-posterior axis of the cortex, corresponding to the positions of the coronal sections illustrated in Figure 3.10. For each animal, three serial sections at each position were used for cell counting and the numbers from equivelent bins combined. Only three bins are present in graph C compared to seven bins in graph A, because the dorsal-ventral thickness of the medial wall of the telencephalon decreases towards the caudal edge of the cortex. This can be seen in Figure 3.10. W*nt8B*$^{+/+}$ data denoted by open bars.

3.5 Discussion

The expression pattern of *Wnt8B* in the mouse compares closely with the expression pattern found in other species (Lako et al, 1998; Hollyday et al, 1995; Kelly et al, 1995). The conserved nature of this expression pattern suggests that *Wnt8B* plays an important role in the development of this region. Defining the factors which regulate the development of the medial telencephalic wall is complicated by the large number of candidate genes that are expressed in this region, many in graded and overlapping patterns. This is further complicated by the potential for interaction both between and within these genes families. For example, in studies where Wnt and BMP proteins were injected into *Xenopus* embryos, ectopic head and forebrain structures were induced which were not seen with single injections of either Wnt or BMP protein alone (Glinka et al, 1997). More recently, it has been found that continual Wnt signalling blocks the response of epiblast cells to FGF signals, permitting the expression and signalling of BMP to direct an epidermal fate whereas conversely, a lack of exposure of epiblast cells to Wnt signals permits FGF to induce a neuronal fate (Wilson et al, 2001). As previously described, within the Wnt gene family, members are able to signal through three different pathways which can produce distinct results in terms of cell fate, polarity, proliferation and adhesion.

At E12 and E14 the predominating processes in the development of the medial telencephalic wall are cell proliferation and cell fate determination. Cell polarity may still play a role, though it appears to be more crucial at earlier stages in patterning of the

neural tube (Kiecker and Niehrs, 2001). Inevitably, multiple signalling pathways will be employed to drive these processes. The Wnt/β-catenin pathway may be activated at this time to drive cell fate and proliferation, as members of the *Wnt1* class, including *Wnt3A, Wnt7A* and *Wnt8B* are present in the medial telencephalic wall. Direct evidence for this is seen in a *Wnt3A*$^{-/-}$ mutant which lacks a hippocampus (Lee et al, 2000) and a *Lef-1*$^{-/-}$ mutant which lacks the granule cell layer of the dentate gyrus (Galceran et al, 2000). The Wnt/Ca^{2+} pathway may also be activated at this time as *Wnt5A* is strongly expressed in the medial telencephalic wall, and this may have consequences for cell proliferation in this region, a process that this gene has been shown to regulate in other structures (Yamaguchi et al, 1999).

Crucially, the expression of Frizzled receptors will determine which pathways are activated. Transcripts of *Fz5*, *Fz8, Fz9* and *Fz10* are present in the medial telencephalic wall in addition to the secreted frizzled related proteins (SFRP) SFRP-1 and SFRP-3 which have been found to inhibit Wnt signalling (Kim et al, 2001). Recently, *Wnt8B* was found to activate the Wnt/β-catenin pathway in Xenopus by acting through *Fz7*, though *Fz7* expression has not been reported in the medial telencephalic wall (Sumanas et al, 2000).

Effects of *Wnt8B* on morphogenesis in the medial telencephalic wall at E12 and E14

The results of this study showed that at E12 and E14 no obvious effect on tissue morphogenesis was detectable in brain sections from *Wnt8B*$^{-/-}$ embryos. Taking into

account slight differences in the plane of section of individual embryos, the shape and timing of the developing medial telencephalic wall and cell architecture within it, *Wnt8B*$^{-/-}$ embryos were consistent with that of wild type embryos in these respects. It also appeared that cell density, cortical lamination and therefore migration was normal in *Wnt8B*$^{-/-}$ embryos. Additionally, there were also indications that cell fate was unaffected in *Wnt8B*$^{-/-}$ embryos as at later ages the granule cell layer of the dentate gyrus and pyramidal cells of all CA regions of the hippocampus were clearly present.

Effects of *Wnt8B* on cell proliferation in the medial telencephalic wall at E12 and E14

The results of this study show that proliferative rates at E12 and E14 are not altered in *Wnt8B*$^{-/-}$ embryos compared to those in *Wnt8B*$^{+/+}$ embryos. Given the striking phenotypes generated in other Wnt knockouts it is perhaps surprising that none are apparent in this mutation.

One possible explanation for this is that the *Wnt8B*$^{-/-}$ allele used here is not a true null allele (i.e. it is still able to express functional protein). Figure 3.1 illustrates the Wnt8B amino acid sequence and indicates the deleted region in *Wnt8B* homozygous mutants compared to other *Wnt* mutants. Most *Wnt* family members contain four exons with the conserved cystiene residues clustered mainly in the last two exons. *Wnt8B* contains six exons, and in the homozygous mutant the last three exons are deleted. Exons four, five and six of *Wnt8B* include the sequences for conserved cysteine residues. Table 3.1 compares how other *Wnt* knockouts have been made in the mouse, and describes the resultant phenotypes. This table shows that most comonly, *Wnt* knockouts have been

produced by an insertion mutation around exons two and three. Additionally, the deletion of a single base pair (in Wnt1) has also proved sufficient to produce a non-fuctional Wnt protein (producing a frame shift in translation of the gene)(Thomas et al, 1991), and it has been shown *in vitro,* that the substitution of serine for two of the conserved cysteine residues is sufficient to produce a non-functional protein (though substitution of cysteins at two other sites had no functional effect) (Mason et al, 1992). Taken together these data strongly suggest that the loss of two thirds of the normal Wnt8B protein, should be sufficient in producing non fuctional Wnt8B protein in these mice. Since no antibody to *Wnt8B* is available, the nature of any protein produced from the mutant allele cannot be determined.

One reason a more obvious phenotype is not seen may be the number of other *Wnt* genes which are expressed in an overlapping pattern to *Wnt8B* throughout embryonic development. It is tempting to speculate that where two or more genes are present performing the same function, such as controlling proliferation for example, the effects of the absence of one of them may be compensated for by the others. This effect has been found in a compound mutants of $Wnt1^{-/-}/Wnt3A^{-/-}$, which has a reduction in dorsolateral precursor cell number, not seen in either $Wnt1^{-/-}$ or $Wnt3A^{-/-}$ mutants alone (Ikeya et al, 1997). Additionally, two members of the *Dlx* gene family *Dlx-1* and *Dlx-2* have almost identical expression patterns in the striatum from E9.5. In $Dlx\text{-}1^{-/-}$ and $Dlx\text{-}2^{-/-}$ mutants, striatal histogenesis proceeds as normal, however, in $Dlx\text{-}1^{-/-}Dlx\text{-}2^{-/-}$ double knockout mice this process is disrupted (Anderson et al, 1997).

3.6 Conclusion

In the absence of *Wnt8B,* no alteration was observed on cell proliferation or morphology compared to wild type animals at two embryonic ages using BrdU analysis and Nissel staining. Since only two ages were studied an effect on proliferation can not be excluded, but is unlikely. It may be that the function of *Wnt8B* is in directing cell fate. Alternitively, other *Wnt* genes which are expressed in overlapping patterns with that of *Wnt8B* may act to compensate for its absence.

To investigate cell fate in *Wnt8B*$^{-/-}$ embryos, immunohistochemical labelling for selective markers of neuronal and glial cell types could be carried out at developmental stages leading on from E14 when the secondary population of glial cell progenitors are starting to produce post mitotic cells, and the process of cell differentiation becomes more important.

To investigate the possibility that other *Wnt* genes are compensating for *Wnt8B* compound mutants could be generated similar to the *Wnt1*$^{-/-}$*Wnt3A*$^{-/-}$ mutants which have a reduction in dorsolateral precursor cell number, not seen in either *Wnt1*$^{-/-}$ or *Wnt3A*$^{-/-}$ mutants alone (Ikeya et al, 1997). *In vitro* analysis may also be used to further investigate the effects of *Wnt8B* by allowing a much greater range of manipulations. For example, cell lines transfected with *Wnt8B* or a combination of *Wnt8B* and other Wnt genes and Frizzled receptors, such as those also found in the medial wall of the telencephalon, could be used to determine selective effects on cell fate and proliferation.

The expression pattern of *Wnt8B* is tightly restricted and highly evolutionarily conserved which implies that it is necessary to the development of this region. It is possible that *Wnt8B* is responsible for a more subtle effect such as axon guidance or the development of a specific cell type which can not be detected by these methods or does not occur at the time points chosen for analysis.

CHAPTER 4. The role of *Wnt8B* in neurogenesis in the adult dentate gyrus

4.1 Abstract

In situ hybridisation for *Wnt8B* was carried out on sections of adult mouse brain to determine if *Wnt8B* was expressed in maturity. Specific domains of expression were identified in a number of tissues and cell types such as the cells of the sub granular layer of the dentate gyrus.

The expression of genes involved in Wnt signalling has been increasingly observed in adult animals but their role is unknown. In developing embryos much evidence shows that many members of the *Wnt* gene family are involved in cell proliferation. Excitement has recently been generated by the discovery of adult neurogenesis in the cells of the lateral ventricles and the sub granular layer of the dentate gyrus.

BrdU labelling experiments were carried out in $Wnt8B^{-/-}$ and $Wnt8B^{+/+}$ animals to determine whether *Wnt8B* plays a role in cell proliferation in the cells of the dentate gyrus. A number of experimental paradigms were used to give short and long pulses of BrdU. Although preliminary results from BrdU studies indicated that in the absence of Wnt8B cell proliferation may be reduced, these studies proved difficult to interpret due to the potential for many factors such as sex, environmental stimulation, and method of BrdU delivery to influence the experimental outcome. Building on these experiments, the experimental conditions were modified to attempt to confine the outcome of a BrdU labelling assay to the effects of the gene alone. Finally, a tightly controled, cumulative

BrdU labelling study was carried out in *Wnt8B*$^{-/-}$ and *Wnt8B*$^{+/+}$ animals, and cell proliferation in the dentate gyrus was determined at subsequent time points. No proliferative difference was found.

4.2 Introduction

In vivo, newly generated subgranular zone cells (in adult mice) begin to express neuron specific markers within a few days of their birth and demonstrate morphological features characteristic of dentate granule cells including contributing to mossy fibre projections to the CA3 area of the hippocampus (Gage et al, 1995).

As described in Chapter 1, proliferation of these cells is regulated by many factors such as hormones, neurotransmitters, growth factors and environmental stimulation, and it has become clear that the proliferative rates of adult hippocampal progenitors (AHPs) at any given time will reflect environmental conditions. Once born, other regulatory factors must be needed to enable these cells to survive, migrate, differentiate and integrate into their new surroundings. These cells maintain the properties of embryonic cells in their abilities to carry out these processes, and it is likely that similar factors facilitating these processes during embryonic development may act on these cells during adulthood. Based on cultures of adult hippocampal cells, prime candidates for these factors are growth factors such as FGF-2 and trophic factors such as BDNF which exert proliferative and differentiative effects on these cells respectively (Ray et al, 1993; Takahashi et al, 1999).

Recently, a number of studies have suggested that *Wnt* genes may play a role in the adult brain. In adult mice, a gene targeting study of *dishevelled-1* (a component of the Wnt pathway) has revealed that it influences social behaviour and sensorimotor gating (Lijam et al, 1997; Geyer & Braff, 1987). These have been suggested key factors in several human psychiatric disorders. The expression of *Wnt1* has been found in adult human brains in the CA3 and CA4 region of the hippocampus and is known to be elevated in schizophrenic patients (Miyaoka et al, 1999), a disorder previously linked to abnormalities in Wnt signalling (Cotter et al, 1998). It has been proposed that these effects may be related to cell adhesion, synaptic rearrangement and plasticity (McMahon & Bradley, 1990; Parr et al, 1993).

The elucidation of the role of *Wnt* genes in the adult brain is at an early stage as most previous research has been directed towards embryogenesis. Taken together, these studies indicate that the dysfunction of Wnt signalling in the adult brain may lead to psychiatric disorders. A better understanding of the role of Wnt signalling in the adult brain may be necessary in developing therapies for these conditions. To achieve this, further research is needed to determine whether other members of the *Wnt* gene family are expressed during adulthood and their roles in normal brain function.

4.3 Aims and Methods

Aims

i) To determine the detailed expression pattern of *Wnt8B* in the adult brain

ii) To determine any effects of loss of *Wnt8B* on proliferation in the dentate gyrus

iii) To evaluate different BrdU protocols

Methods (for detailed methods see Chapter 2)

In situ hybridisation for *Wnt8B* in the adult brain

In situ hybridisation using a digoxygenin labelled probe for *Wnt8B* was carried out on coronal wax sections from two month old mice. No alteration in the *in situ* hybridisation protocol used for embryonic sections was made for adult tissue. The *Wnt8B* cDNA plasmid was linearised with Xba and transcribed (using Boehringer Mannheim DIG RNA labelling kit, following the manufacturers instructions) with T3 RNA polymerase (antisense), or T7 RNA polymerase (sense) which gave no signal (data not shown).

BrdU labelling experiments

Experiment a) seven day BrdU labelling

To examine any effect of *Wnt8B* on cell proliferation in the dentate gyrus, three pairs of $Wnt8B^{-/-}$ and $Wnt8B^{+/+}$ or $Wnt8B^{+/-}$ mice were given a single injection of BrdU (i.p.) each day for seven days and sacrificed 24 hours after the last injection.

Experiment b) one day BrdU labelling

To examine any effect of *Wnt8B* on cell death of newly born cells in the dentate gyrus, three pairs of $Wnt8B^{-/-}$ and $Wnt8B^{+/+}$ or $Wnt8B^{+/-}$ or mice were given a single injection of BrdU (i.p.) and sacrificed 5 hours after the last injection.

Experiment c) 48 hour cumulative BrdU labelling

To examine any effect of *Wnt8B* on cell death and cell survival of newly born cells in the dentate gyrus by cumulative BrdU labelling via drinking water, six pairs of age and sex matched $Wnt8B^{+/+}$ and $Wnt8B^{-/-}$ littermates were housed in separate cages under identical conditions. Three days later the pairs were given BrdU supplied in drinking water over a 48 hour period. Three pairs were sacrificed immediately at the end of the labelling period, and the remaining three pairs were sacrificed seven days later.

Experiment d) 12 hour cumulative BrdU labelling and time course study

To examine the time course of any effect of *Wnt8B* on cell death and cell survival of newly born cells in the dentate gyrus, by cumulative BrdU labelling via injection, 12 pairs of age and sex matched $Wnt8B^{+/+}$ and $Wnt8B^{-/-}$ littermates were housed in separate cages under identical conditions. Three days later each animal received an injection of BrdU (i.p.) at two hour intervals over a 12 hour period. Two hours after the last injection was deemed time point 0. Three pairs of $Wnt8B^{-/-}$ and $Wnt8B^{+/+}$ littermates were subsequently sacrificed at timepoints: 0, 12, 24, 48 and 72 hours.

BrdU labelling analysis

Brains were wax embedded and sectioned coronally at 10μm. Every tenth section along the anterior-posterior axis of the hippocampus was used for BrdU

immunocytochemistry. In each section the number of BrdU labelled cells lying within the layers of the subgranular zone, granular zone, CA fields, and hilus were counted (cells were deemed either positive or negative for BrdU). Labelled cells in the area encircled by the dentate gyrus and CA fields (termed Z) were also counted. (see Figure 4.1). The labelling index was not determined as in Chapter three since it was difficult to identify numbers of non labelled cells due to the nature of the tissue.

The mean of the data was used to generate bar graphs showing the distribution of BrdU labelled cells in *Wnt8B*$^{-/-}$ and *Wnt8B*$^{+/+}$ animals. Statistical differences between *Wnt8B*$^{-/-}$ and *Wnt8B*$^{+/+}$ animals were made using the student's *t*-test and values expressed as $\pm$ S.E.M. (*= $P<0.1$, **=$P<0.01$). In Figures 4.4-4.7, BrdU labelling is presented as the mean number of BrdU labelled cells per 10μm section, per area of the hippocampus and dentate gyrus. In Figure 4.8, BrdU labelling is presented as the mean number of BrdU labelled cells per 10μm section, per area of the hippocampus and dentate gyrus at different time points after the end of the BrdU labelling period. This data is shown for male and female mice of each genotype, plus data combined for male and female mice of the same genotype. In Figure 4.9, BrdU labelling is presented as the mean number of BrdU labelled cells per 10μm section, in sections every 100μm along the rostro-caudal axis of the hippocampus. In Figure 4.10, BrdU labelling is presented as the mean number of BrdU labelled cells in *Wnt8B*$^{-/-}$ and *Wnt8B*$^{+/+}$ animals, per area of the hippocampus and dentate gyrus.

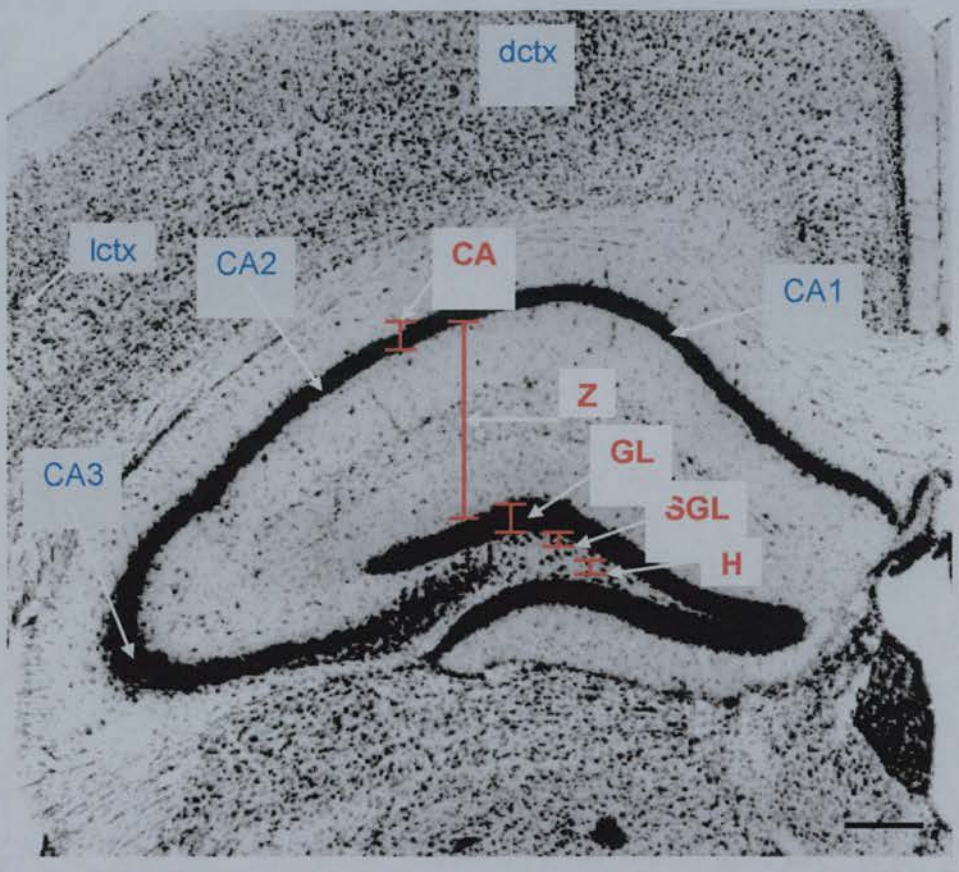


Figure 4.1 Photomicrograph of a coronal section of the hippocampus and dentate gyrus, illustrating the cell layers which comprise these structures, and the areas (indicated by red bars) used for cell counting. BrdU labelled cells were designated as falling within the CA area if they appeared within the pyramidal cell layer of either of the CA fields. Cells bordering this area were not counted in this category. Similarly, BrdU labelled cells were designated to the areas of subgranular and granular cell layers. BrdU labelled cells were designated to the hilus (H) and Z category, if they lay within the areas bordered by the subgranular layer, and the granular layer and CA fields, respectively. Photomicrograph reproduced from Paxinos et al, (1994). Abbreviations: dctx, dorsal cortex; lctx, lateral cortex;GL, granule cell layer; SGL, subgranular cell layer; H, hilus. Scale bar, 50µm

4.4 Results

4.4.1 *Wnt8B* expression in the adult brain

To detect transcripts of *Wnt8B* on coronal sections, *in situ* hybridisation was used. *In situ* hybridisation shows that *Wnt8B* is expressed in multiple sites and in restricted domains in the adult brain. *Wnt8B* is strongly expressed in the cingulate cortex, the subgranular layer of the dentate gyrus, the Purkinje cell layer of the cerebellum (Figure 4.2), and in the motor cortex, CA2 and CA3 region of the hippocampus (data not shown). *Wnt8B* expression in the cingulate cortex appeared to be restricted to a group of cells in layers two and three of the lateral cortical wall. In coronal sections from different positions along the anterior-posterior axis, the dorso-ventral level of this expression appeared to change. This may be due to a restricted expression in a set of cells which similarly appear at slightly different dorso-ventral positions. High levels of expression were visible throughout the purkinke cell layer of the cerebellum and in the subgranular cell layer of the dentate gyrus. Weaker levels of expression were also found in the CA2 and CA3 pyramidal cell layer of the hippocampus and in the anterior-medial most aspect of the cerebral cortex.

Due to the involvement of *Wnt* genes in cell proliferation (Moon et al, 1997), and the recently confirmed neurogenic property of the subgranular cells of the dentate gyrus (Gage et al, 1995), BrdU labelling studies were carried out in *Wnt8B*$^{+/+}$, *Wnt8B*$^{+/-}$ and *Wnt8B*$^{-/-}$ mice to determine whether the absence of Wnt8B signalling may affect cell proliferation in this area (see Figure 4.3).

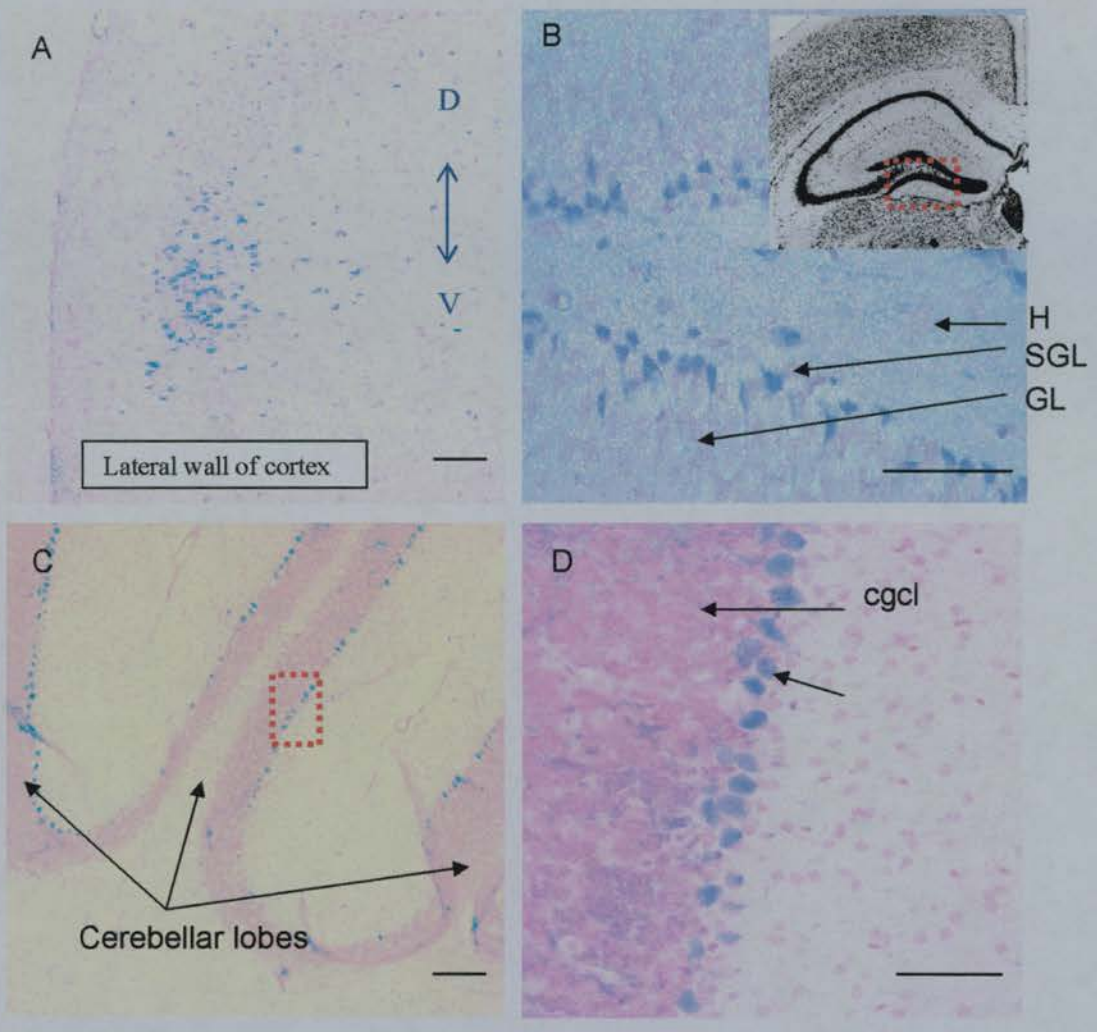


Figure 4.2 *In situ* hybridisation for *Wnt8B* in coronal sections from adult brain. (A) A restricted domain of expression of *Wnt8B* in layers two and three of the cingulate cortex. (B) *Wnt8B* is strongly expressed in the subgranular layer of the dentate gyrus (inset of low power image of dentate gyrus indicates area (red box) shown). (C) High levels of *Wnt8B* expression are found in the lobes of the cerebellum. (D) A high power view of the Purkinje cell layer of the cerebellum, showing specific expression of *Wnt8B* in the Purkinje cells (red box in C indicates area shown). *Wnt8B* expressing cells denoted by a purple colour. Abbreviations: D, dorsal; V, ventral; pcl, Purkinje cell layer; cgcl, cerebellar granule cell layer. Scale bars, 50μm.

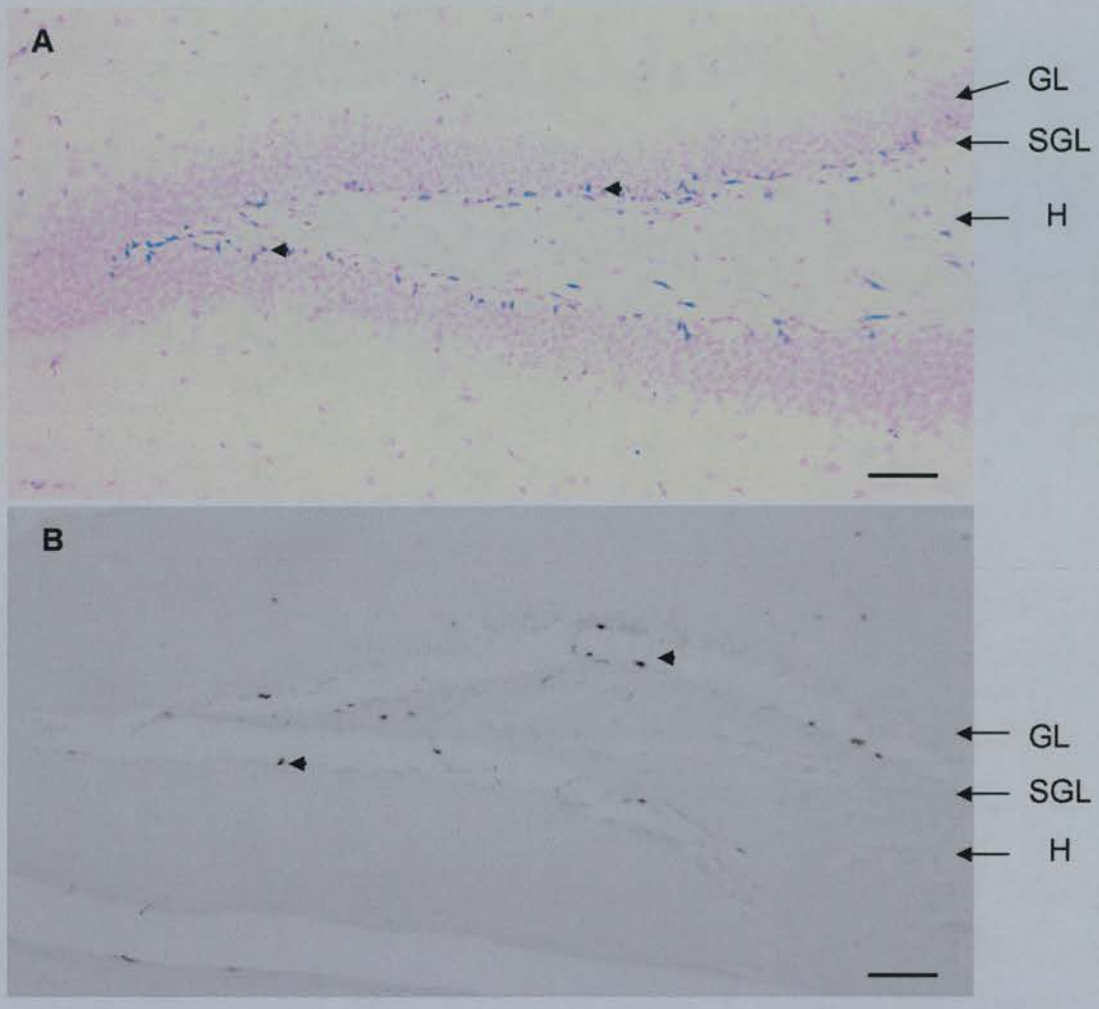


Figure 4.3 Cells of the subgranular layer of the dentate gyrus express *Wnt8B* and are a site of continuing cell proliferation in the adult. (A) *In situ* hybridisation for *Wnt8B* in a coronal section of the dentate gyrus, clearly indicates strong expression throughout the subgranular layer of the dentate gyrus (arrow heads). (B) Immunocytochemistry for BrdU in a matched section, revealing new cell proliferation in the subgranular layer (arrow heads). Abbreviations: GL, granule cell layer; SGL, subgranular cell layer; H, hilus. Scale bars, 50μm.

4.4.2 BrdU labelling of newly born cells in the hippocampus and dentate gyrus in *Wnt8B*$^{+/+}$ or *Wnt8B*$^{+/-}$ and *Wnt8B*$^{-/-}$ mice.

Experiment a) Seven day BrdU labelling

In a preliminary experiment to examine any effect of *Wnt8B* on cell proliferation in the dentate gyrus, three pairs of *Wnt8B*$^{-/-}$ and *Wnt8B*$^{+/+}$ or *Wnt8B*$^{+/-}$ mice (3 month old) were given a single injection of BrdU (i.p.) each day for seven days and sacrificed 24 hours after the last injection. As adult animals were used in which the rates of cell proliferation were thought to be relatively low, and because of the short half life of BrdU in vivo (approximately two to four hours), a number of BrdU injections were administered in an attempt to label a larger number of cells than would be expected from a single injection alone. *Wnt8B*$^{+/-}$ mice were used along with *Wnt8B*$^{+/+}$ mice as controls where *Wnt8B*$^{+/+}$ mice were not available, and because none of the published *Wnt* knockouts have been reported to possess a heterozygous phenotype.

The results show the mean number of BrdU labelled cells counted per 10μm section, in three adult *Wnt8B*$^{-/-}$ mice and three *Wnt8B*$^{+/+}$ or *Wnt8B*$^{+/-}$ mice of various ages and sexes and which were held in shared cages. As illustrated in Figure 4.1 numbers of BrdU labelled cells were counted in the granule cell layer, hilus, CA fields, Z area and subgranular cell layer in every tenth section through the anterior-posterior axis of the hippocampus. No difference in BrdU labelling was found in the granule cell layer, hilus, CA fields, or Z area. However, in the subgranular cell layer the numbers of BrdU

labelled cells in *Wnt8B*$^{+/+}$ or *Wnt8B*$^{+/-}$ animals was found to be significantly higher than in *Wnt8B*$^{-/-}$ mice (Figure 4.4).

This finding was consistent with the hypothesis that Wnt8B signalling may be involved in regulating cell proliferation in this area. These findings indicate that after a long period of BrdU labelling, fewer cells are labelled in the subgranular layer of the dentate gyrus in *Wnt8B*$^{-/-}$ animals. This may reflect a decrease in proliferation, increased cell death, or a combination of both possibilities. To attempt to distinguish between these three possibilities, a short BrdU labelling study was carried out (see Experiment b).

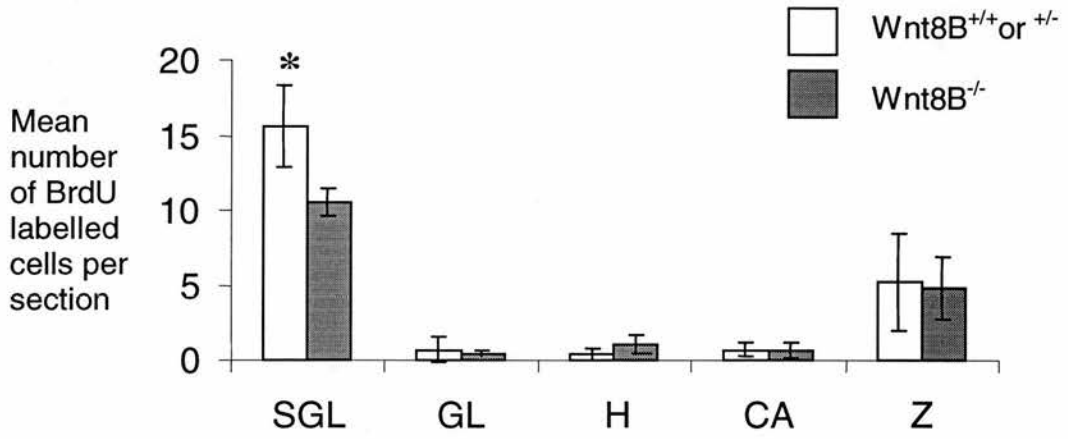


Figure 4.4 Graph illustrating the mean number of BrdU labelled cells per 10μm section (in 15 sections), per area of the hippocampus and dentate gyrus (± s.e.m)(*$P <$ 0.1), in *Wnt8B*$^{+/+}$ or *Wnt8B*$^{+/-}$ and *Wnt8B*$^{-/-}$ mice, after a single pulse of BrdU was injected each day for seven days. Abbreviations: GL, granule cell layer; SGL, subgranular cell layer; H, hilus.

Experiment b) One day BrdU labelling

To attempt to distinguish whether the decrease in BrdU labelling found in Experiment a) was the result of an effect of *Wnt8B* on cell death or proliferation of newly generated subgranular cells, a short BrdU labelling study was carried out. As it is unlikely that newly generated cells would die within a short time period, a single injection of BrdU was given to three *Wnt8B*$^{+/+}$ or *Wnt8B*$^{+/-}$ and three *Wnt8B*$^{-/-}$ mice of various ages and sexes and which were held in shared cages.

The mean numbers of BrdU labelled cells counted per 10μm section, five hours after the injection, showed no difference in labelling (in any of the areas analysed) between *Wnt8B*$^{+/+}$ or *Wnt8B*$^{+/-}$ and *Wnt8B*$^{-/-}$ animals (Figure 4.5). This result suggested that the difference in the numbers of BrdU labelled cells found in *Wnt8B*$^{+/+}$ or *Wnt8B*$^{+/-}$ and *Wnt8B*$^{-/-}$ animals in the subgranular zone, in Experiment a), may not be accounted for by cell proliferation, (as this difference would still be expected to be reflected over a short period of BrdU labelling).

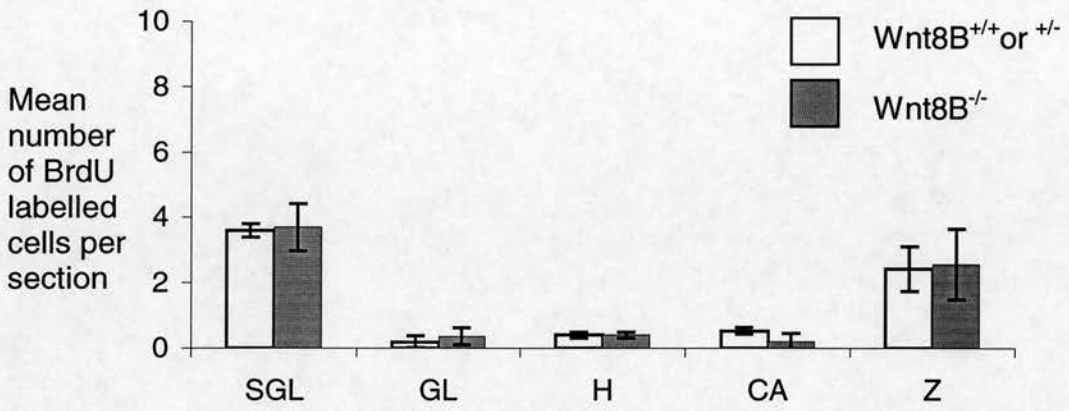


Figure 4.5 Graph illustrating the mean number of BrdU labelled cells per 10μm section, per area of the hippocampus and dentate gyrus (± s.e.m), in *Wnt8B*$^{+/+}$ or *Wnt8B*$^{+/-}$ and *Wnt8B*$^{-/-}$ mice, five hours after a single pulse of BrdU was injected. Abbreviations: GL, granule cell layer; SGL, subgranular cell layer; H, hilus.

Experiment c) 48 hour cumulative BrdU labelling

As the cell proliferation in the subgranular layer may be influenced by many different factors (See Chapter 1), an experiment was designed to attempt to restrict the outcome to the effect of *Wnt8B* alone. BrdU was supplied in drinking water in order to cumulatively label newly generated cells. This enabled the continuous bioavailability of BrdU compared to Experiments A) and b), where many cells born at times between injections will not have been exposed to BrdU. To control for the effects of age, sex and social interaction, individually caged, sex matched littermates of each *Wnt8B* genotype were compared in this analysis.

After a 48 hour period of BrdU administration, and seven days after the 48 hour period of BrdU administration, no differences between $Wnt8B^{+/+}$ and $Wnt8B^{-/-}$ animals in the mean number of BrdU labelled cells counted per 10μm section was seen (Figure 4.6, 4.7).

Subsequent to this experiment, I learned that the method of BrdU administration (i.e. via drinking water) was unreliable because individual mice drink different amounts of water, and at different times over a 24 hour period. Therefore the results of this experiment could not be relied on as accurate. For example, if $Wnt8B^{-/-}$ animals drank less than their wild type counterparts, proliferation would be underestimated. Therefore, a second cumulative labelling experiment was carried out using injection as the BrdU delivery method (see Experiment d).

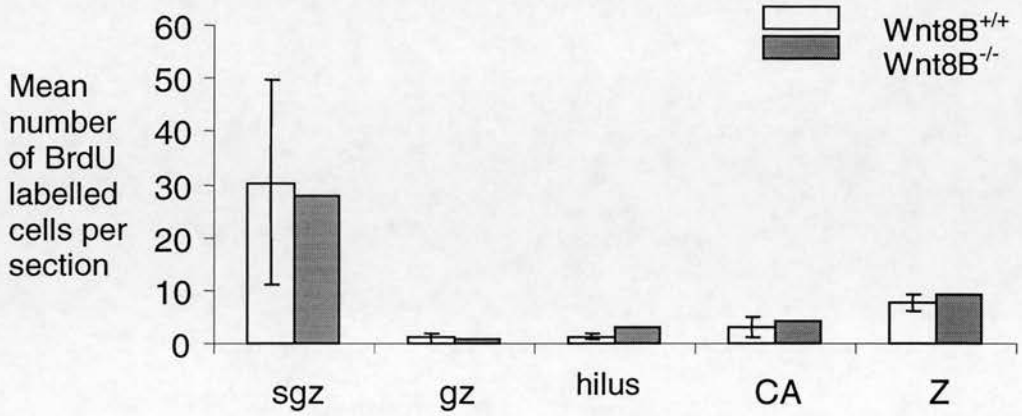


Figure 4.6 Mean number of BrdU labelled cells (± s.e.m) in the hippocampus and dentate gyrus in three pairs of *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ littermates, two hours after a 48 hour period of BrdU administration via drinking water. Abbreviations: GL, granule cell layer; SGL, subgranular cell layer; H, hilus.

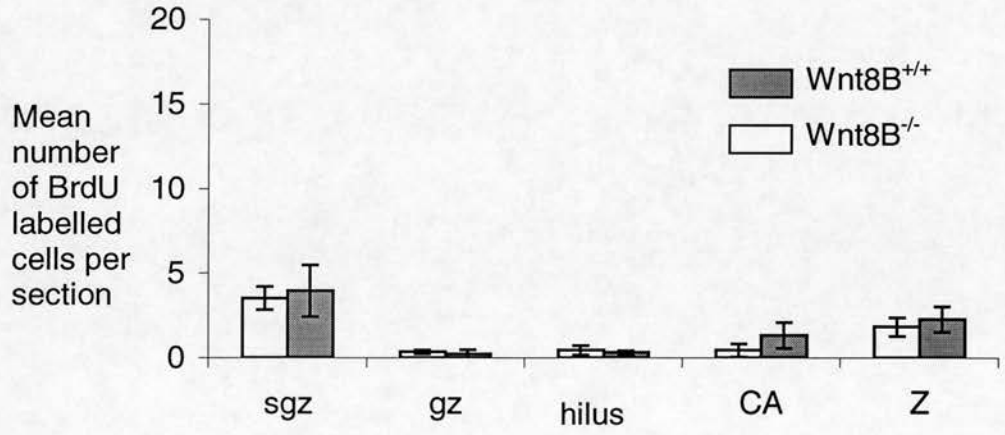


Figure 4.7 Graph illustrating the mean number of BrdU labelled cells per 10µm section, per area of the hippocampus and dentate gyrus (± s.e.m), in three pairs of individually caged *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ littermates, seven days after a 48 hour period of BrdU administration via drinking water. Abbreviations: GL, granule cell layer; SGL, subgranular cell layer; H, hilus.

Experiment d) 12 hour cumulative BrdU labelling and time course study

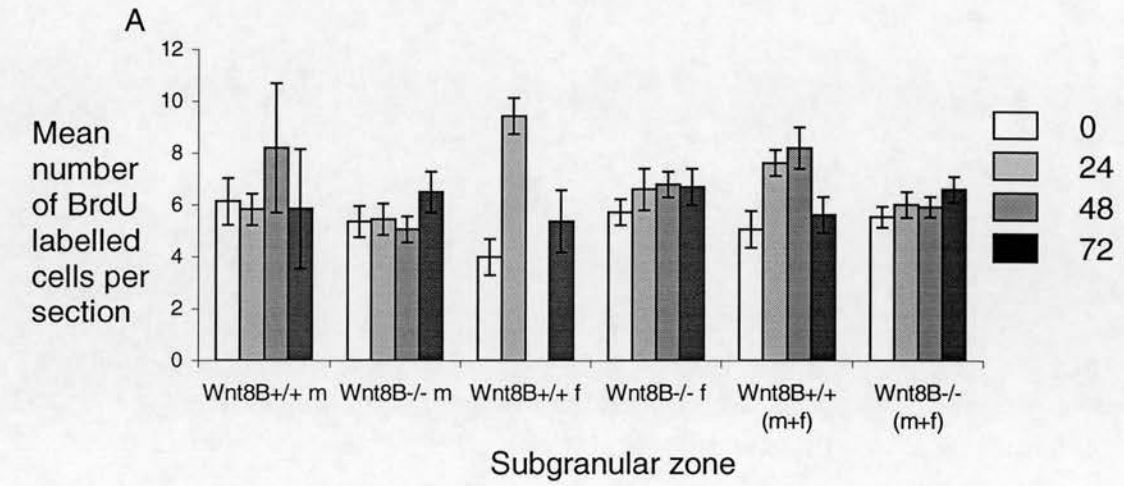
The cumulative labelling study carried out in Experiment c) to distinguish between the effects of *Wnt8B* on cell death and cell proliferation, under rigorously controlled experimental conditions, was found to be unreliable. Due to this a further experiment was designed using BrdU injection rather than administration through drinking water.

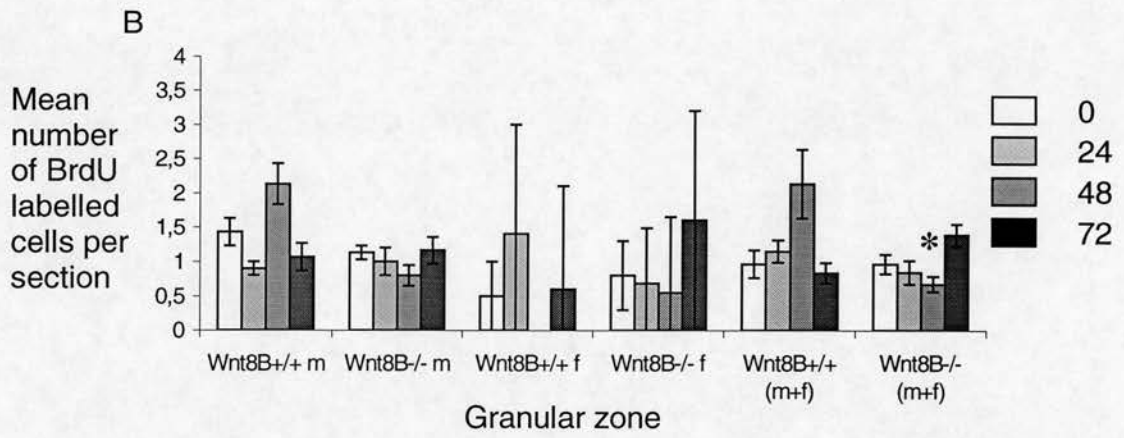
Twelve pairs of $Wnt8B^{+/+}$ and $Wnt8B^{-/-}$ littermates which were controlled for age (2 months old), sex (paird littermates were always same sex) and social interaction received an injection of BrdU (i.p.) at two hour intervals over a 12 hour period. Two hours after the last injection was deemed time point 0. Three pairs of $Wnt8B^{-/-}$ and $Wnt8B^{+/+}$ littermates were subsequently sacrificed at timepoints: 0, 12, 24, 48 and 72 hours (n=3 pairs of $Wnt8B^{+/+}$ and $Wnt8B^{-/-}$ littermates at each time point)(See appendix 2 for raw data).

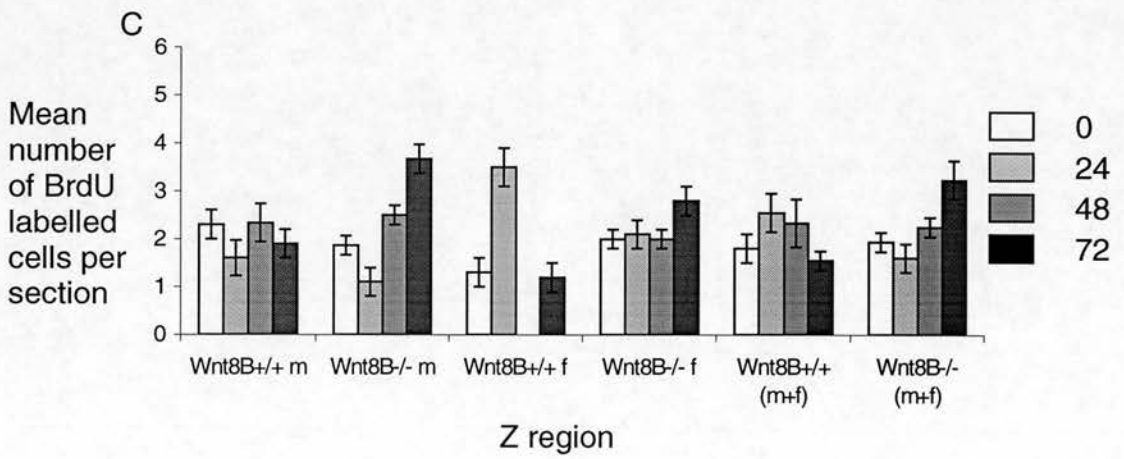
The mean number of BrdU labelled cells (per 10μm section) in each of the counting areas, for male and female littermates (along with the combined data for males and females) are displayed against time after the end of the labelling period (Figure 4.8). In all cases no significant differences in the numbers of labelled cells were found.

Additionally, data for individual pairs of $Wnt8B^{+/+}$ and $Wnt8B^{-/-}$ littermates were compared (data not shown). The profile of the mean number of labelled cells (per 10μm section) (combined data from all $Wnt8B^{+/+}$ and $Wnt8B^{-/-}$ animals sacrificed at 0, 24, 48

and 72 hours after end of the labelling period), along the anterior-posterior axis were also determined, to investigate any axial differences in BrdU cell labelling (Figure 4.9). The combined data for the numbers of BrdU labelled cells in each area counted, from *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ animals were also determined (Figure 4.10). In all cases, no significant differences in the numbers of labelled cells were found.


A
Mean number of BrdU labelled cells per section
12
10
8
6
4
2
0
0
24
48
72
Wnt8B+/+ m
Wnt8B-/- m
Wnt8B+/+ f
Wnt8B-/- f
Wnt8B+/+ (m+f)
Wnt8B-/- (m+f)
Subgranular zone


B
Mean number of BrdU labelled cells per section
4
3,5
3
2,5
2
1,5
1
0,5
0
*
0
24
48
72
Wnt8B+/+ m
Wnt8B-/- m
Wnt8B+/+ f
Wnt8B-/- f
Wnt8B+/+ (m+f)
Wnt8B-/- (m+f)
Granular zone


C
Mean number of BrdU labelled cells per section
6
5
4
3
2
1
0
0
24
48
72
Wnt8B+/+ m
Wnt8B-/- m
Wnt8B+/+ f
Wnt8B-/- f
Wnt8B+/+ (m+f)
Wnt8B-/- (m+f)
Z region

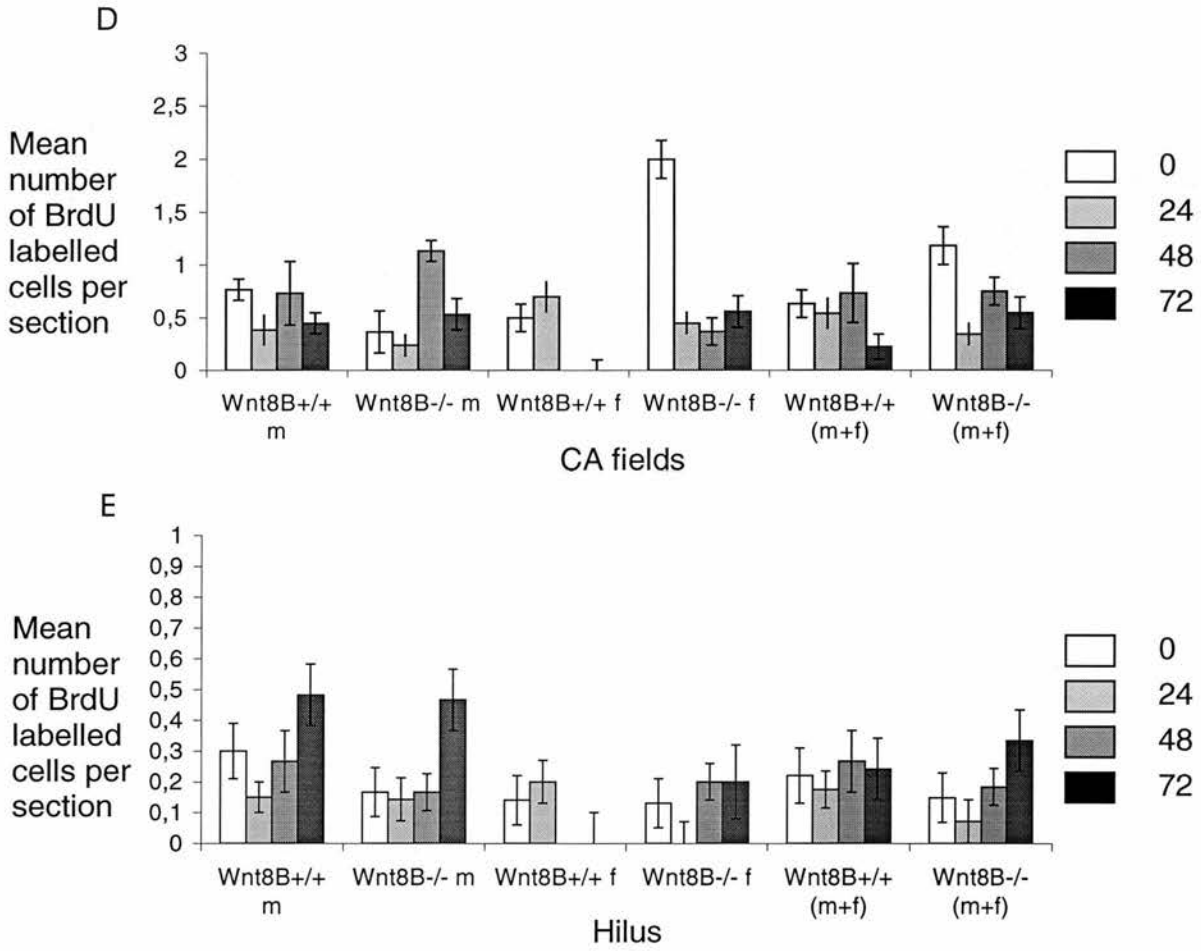


Figure 4.8 Mean number of BrdU labelled cells per 10μm section (± s.e.m) (*$P <$ 0.1), in the hippocampus and dentate gyrus in male and female *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ littermates and in male+female *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ animals, at periods of 0, 24, 48 and 72 hours after cumulative BrdU labelling by BrdU injection every two hours for 12 hours (n=3 pairs of in *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ littermates at each time point). Mean number of BrdU labelled cells are shown for the subgranular cell layer of the dentate gyrus, granular cell layer, Z region, CA fields, and Hilus, (A-E) respectively.

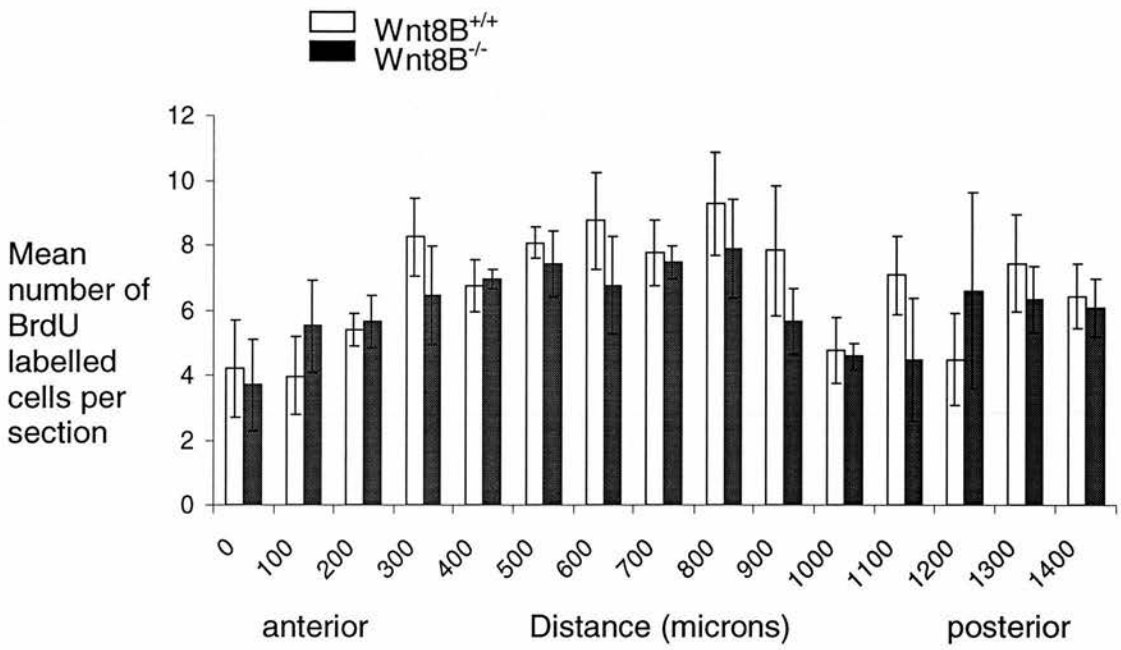


Figure 4.9 Graph showing mean number (± s.e.m) of BrdU labelled cells from each of the fifteen sections taken from along the anterior-posterior axis of the hippocampus, from twelve pairs of *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ littermates, after cumulatively labelling with BrdU by injection every two hours for 12 hours. A student's *t*-test found no significant differences between *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ data (P=0.37). Cell counts from sections for all animals used in experiment d were used, and the combined data for *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ animals is shown.

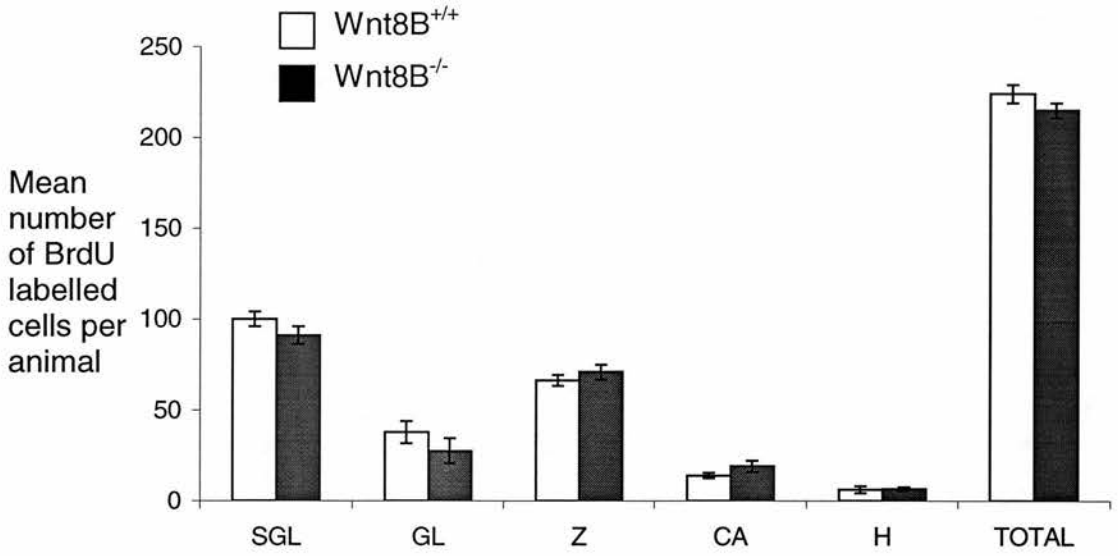


Figure 4.10 Graph showing the overall mean number (+ s.e.m) of BrdU labelled cells per animal, in *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ mice, in each area of the hippocampus, after cumulatively labelling with BrdU by injection every two hours for 12 hours. The data combined that from twelve pairs of in *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ littermates sacrificed at 0, 24, 48 and 72 hours after end of the labelling period. Abbreviations: GL, granule cell layer; SGL, subgranular cell layer; H, hilus.

4.5 Discussion

Wnt8B was found to be strongly expressed in restricted domains in the adult brain. *Wnt8B* expression in the adult appears in structures (i.e. hippocampus and dentate gyrus) which are derived from embryonic tissue that expresses *Wnt8B* during development. More surprisingly, *Wnt8B* expression was found in areas of the brain in which expression does not occur during early development, in the anterior dorsal cortex which is involved in motor function, and in the cingulate cortex which is involved in sensory functions. *Wnt8B* expression was also found in the cerebellum, an area of the brain in which *Wnt* genes are necessary for normal development and cell-cell interconnection (McMahon & Bradley, 1990; Hall et al, 2000).

Historically, Wnt genes have been found to play important roles in brain development (see Chapter 1). In adulthood however, many of the processes active during embryogenesis are no longer in action, though the expression of *Wnt* genes and Frizzled receptors is increasingly being found in the adult brain (Katoh, 2002; Kirikoshi et al, 2001; Kirikoshi et al, 2000; Sagara et al, 1998), and in a number of cancer cell lines (Saitoh et al, 2001).

The expression of *Wnt8B* in the subgranular layer of the dentate gyrus suggested a possible role in proliferation in this region. Preliminary BrdU studies carried out in $Wnt8B^{+/+}$ or $Wnt8B^{+/-}$ and $Wnt8B^{-/-}$ mice (Experiments, a and b) indicated that *Wnt8B* expression may be necessary for normal cell survival in this region. Experiment a)

suggests that BrdU labelling in the subgranular layer of the dentate gyrus is decreased in *Wnt8B*$^{-/-}$ mice after seven days labelling by injection. Experiment b) suggests that this is not due to proliferative rates (which may still be expected to be reflected after a short BrdU pulse) but is more likely due to an affect on cell survival.

Experiment c) attempted to control for various factors, such as social interaction, age, stress and gender (which are known to be important in regulating cell proliferation in the subgranular zone, see Chapter 1), which had not been controlled for in Experiment a) or b). In this experiment, age and sex matched littermates were housed separately for three days before the start of a cumulative BrdU labelling experiment, in which BrdU was administered via drinking water, to minimise the effect of stress (from injections) on the mice, and to attempt to label all proliferating cells during the labelling period. Subsequently, this method of BrdU administration was found to be unreliable due to different patterns of drinking behaviour among individual animals.

Finally, a cumulative BrdU pulse duration study was carried out (by BrdU injection, Experiment d), again using age and sex matched littermates which were housed separately prior to the start of the BrdU labelling period. Over a three day time period, no difference in the number of labelled cells were found in *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ mice, similarly to the lack of any proliferative effect seen in Chapter3, (though the combined data for male and females at 48 hours in the subgranular zone was found to be just significant, as determined by a students t-test (P=0.08)). The decreased levels of BrdU labelling in the subgranular layer, which were found in Experiment a) after seven days

labelling are consistent with an affect of *Wnt8B* on cell survival. As the final, cumulative BrdU-time course study did not extend to seven days after the end of the labelling period, this possibility can not be ruled out. However, aspects of this experiment were flawed and on balance, it is more likely that *Wnt8B* is not affecting the survival rates of these cells.

Further studies will be necessary in determining the role of *Wnt8B* in the adult brain. *Wnt8B* expression studies in other species will elucidate whether the expression of this gene is conserved across species, which, may suggest an important role in the adult brain. The expression of *Wnt8B* in the embryo has been found to be conserved (Lako et al, 1998; Kelly et al, 1995). However, a careful study of cell proliferation in the embryo has found no role for *Wnt8B* in neurogenesis in $Wnt8B^{+/+}$ and $Wnt8B^{-/-}$ mice. This suggests that *Wnt8B* may be involved in a different role such as synaptogenesis, or in determining cell fate in the embryo. These possibilities may be investigated more quickly in *in vitro* by transfecting stable cell lines with *Wnt8B* and investigating whether there is any subsequent alteration in cell differentiation by the use of selective markers .

4.6 Conclusion

Multiple restricted domains of *Wnt8B* expression have been identified in the adult forebrain and cerebellum. These sites of expression suggest a role for *Wnt8B* in the adult brain and further studies to determine the expression of related genes such as the *Frizzled* gene family, and to investigate other aspects of cell-cell interaction such as synaptogenesis, will need to be carried out in order to more fully understand the processes which are controlled by Wnt signalling.

Wnt8B alone does not affect the numbers of newly generated cells in the subgranular layer of the dentate gyrus. It is possible that multiple *Wnt* genes are expressed in these cells and the absence of one may be compensated for by another. This possibility may be addresses by producing double knockout animals similarly to those described in Chapter 3. (Ikeya et al, 1997; Anderson et al, 1997).

CHAPTER 5. The role of 2-0-Sulphation of heparan sulphate during forebrain development

5.1 Abstract

Proteoglycans have been proposed as co-factors, necessary for signalling of a range of growth factors. Histological analysis of mice harbouring a gene trap induced mutation in the gene encoding heparan sulphate 2-sulphotransferase (*Hs2st*) indicated a reduction in size of the cerebral cortex (Bullock et al, 1998). To examine whether a change in the rate of proliferation of cortical precursor cells might be a contributing factor, a 5'-bromodeoxyuridine (BrdU) incorporation assay was used to compare the rates of proliferation of precursor cells at E12, and to examine the migration of cortical cells which were born on E13, E15 and E17, in *Hs2st* mutant and wildtype littermate embryos. At E12, a reduction was found in labelling indices of approximately 40% in all cortical areas examined. Cell migration appeared normal, as determined by birthdating analysis, though a significant reduction in cell labelling was found in cells born on E15 and E17. These results indicate that the loss of 2-0-sulphation in one class of proteoglycan, heparan sulphate, leads to a significant reduction in cell proliferation in the developing cerebral cortex.

5.2 Introduction

As described in Chapter 1, heparan sulphate proteoglycans (HSPGs) have an essential involvement in fibroblast growth factor (FGF) signalling in some cell types, where they have been proposed to act as low affinity receptors facilitating the interaction of FGF ligands with high-affinity tyrosine kinase receptors (eg Yayon et al, 1991). In addition, HSPGs are also involved in the Wnt and Sonic hedgehog signalling pathways (Bellaiche et al, 1998; Lin & Perrimon, 1999). The specificity of HSPG-ligand interactions resides, at least in part, in the structure of the heparan sulphate glycosaminoglycan side-chains which vary between cell type and developmental stage in number, length, sequence composition and sulphation pattern (reviewed by Bernfield et al, 1999; Perrimon and Bernfield, 2000; Selleck, 2000)(See Figures 1.8, 1.9). Thus, the regulated synthesis of differentially glycanated proteoglycans represents a potential means to regulate cell-cell communication during development.

The importance of HSPG molecules in embryonic development has been most studied in Drosophila (reviewed by Baeg and Perrimon, 2000). Genes affecting HS synthesis in Drosophila include *sugarless* (*sgl*) which encodes UDP-D-glucose dehydrogenase, loss of which results in complete absence of HSPG molecules and *Sulfateless* (*sfl*), which encodes *N*-deacetylase/*N*-sulphotransferase, an enzyme involved in their post-translational modification. In these mutants, Wingless (*Wg*, a Drosophila *Wnt* gene), and FGF signalling pathways are severely impaired (Lin et al, 1999). Additionally, a third mutation in the Drosophila gene *Tout-velu* (*ttv*) which impairs heparan sulphate production affects hedgehog signalling (Bellaiche et al, 1998; Lin and Perrimon, 1999).

Evidence for tissue-type selectivity of HSPG function in vertebrate development comes from studies of mouse embryos that are homozygous for a gene-trap-induced mutation in the gene encoding a heparan sulphate 2-sulphotransferase (Hs2st) which sulphates heparan sulphate specifically at the 2-O position (Bullock et al, 1998). A gene trap vector was constructed which contained a splice acceptor sequence linked to a lac-Z-neomycin phosphotransferase fusion gene (Skarnes et al, 1995). This vector was allowed to integrate into stem cells. B-gal enzyme activity was detected in one cell line (ST125). Adult chimeras were generated to transmit the integration through the germ line and a line of mice was established heterozygous for the gene trap allele (Bullock et al, 1998). These embryos showed absence of kidney induction and defects of the eye, skeleton and female genital system. The mutation is was found to be homozygous lethal, with homozygotes dying perinatally.

At the biochemical level, the best characterised role for HSPG is in the regulation of FGF and FGF receptor (FGFR) signalling activity. Distinct HS saccharide sequences have been shown to regulate specific FGF-FGFR interactions (Guimond and Turnbull, 1999). Optimal FGF2 binding depends on the presence of 2-0-sulphated heparan sulphate, but activation of the receptor is instead dependent upon 6-0-sulphated heparan sulphate (Guimond & Turnbull, 1999).

The complexity of glycosaminoglycans may provide them with versatile roles in developmental processes. Heparan sulphate proteoglycans have been shown as essential

components of multiple signalling pathways, and understanding their expression and actions will enable a more detailed understanding of their role during early development.

5.3 Aims and Methods

Aims

i) To determine whether anatomical defects occur in the brains of *Hs2st*$^{-/-}$ mice at E12 and E19.

ii) To examine cell proliferation in the dorso-medial wall of the cerebral cortex at E12 in *Hs2st*$^{+/+}$ and *Hs2st*$^{-/-}$ mice.

iii) To examine cell migration in the cerebral cortex of *Hs2st*$^{+/+}$ and *Hs2st*$^{-/-}$ mice.

Methods (for detailed experimental methods see chapter 2.)

5.3.1 Genotyping of *Hs2st* mutants

Mice were from isolated laboratory colonies of C57Bl/6 mice. Embryonic and adult mice were genotyped as described by Bullock et al, (1998).

5.3.2 *LacZ* expression pattern analysis

To detect *lacZ* expression, embryos were dissected from the uterus in M2 medium containing 10% fetal bovine serum (Advanced Protein Products) as described by Hogan et al, (1994) and X-gal-stained overnight according to the method of Beddington et al, (1989).

5.3.3 Anatomical analysis

E19 embryos were processed for wax sectioning and counterstained with Haematoxylin and Eosin using standard techniques. To visualise thalamocortical axons with dioctadecyl tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes, USA), E19 embryos were fixed with paraformaldehyde, small crystals of DiI were placed into the ventro-basal nucleus of the thalamus and, after diffusion, 40μm coronal sections were cut with a vibratome, wet-mounted in PBS, and viewed under rhodamine fluorescence optics.

5.3.4 BrdU labelling

Pregnant dams were given a single injection of BrdU (70μg/g in sterile saline i.p) on either E12, E13, E15 or E17. Mothers were deeply anaesthetised with urethane (0.3 ml of a 25% solution in normal saline i.p) and embryos were removed one hour later, in the case of E12 injected animals, or at E19 in other cases. Homozygous and wild type littermates were identified as described above (Bullock et al, 1998). After dissection, the brains were fixed in 4% paraformaldehyde for two hours at room temperature, embedded in wax, serially sectioned at 10 μm (coronally for E12, parasagitally for E19), mounted on poly-L-lysine coated slides and reacted immunohistochemically to reveal BrdU labelling using the peroxidase method described previously (Gillies and Price, 1993). To estimate proliferative rates, labelling indices (LI) were obtained at each time point (LI: labelled cells as a proportion of total cells; Takahashi et al, 1993). At each time point, three *Hs2st*$^{-/-}$ and three wild type littermates were analysed. Means were

compared statistically using Student's t-test and values expressed as ± S.E.M. (*= $P<0.1$, **=$P<0.01$). In all cases cell counting was carried out blind.

5.4 Results

5.4.1 Anatomical analysis

Tissue sections of E19 *Hs2st*$^{-/-}$ embryos revealed a dramatic reduction in the thickness of the neocortex (Figure 5.1a,b). The hippocampus also appears smaller in the mutant embryo than in the wild type littermate control. The mutation in *Hs2st* was generated by gene-trapping, resulting in the incorporation of a *lacZ* reporter gene into the mutant allele resulting in the production of non functional protein. Expression of the *lacZ* reporter has been shown to closely reflect expression of the endogenous *Hs2st* gene, allowing us to deduce the *Hs2st* expression pattern simply by examining ß-galactosidase activity (Bullock et al, 1998). *Hs2st* is expressed throughout the telencephalon at E12, at the height of neurogenesis, though no expression is seen in the dorsal thalamus which has projections to the neocortex (Figure 5.1c). Similarly, at E19, *Hs2st* expression extends throughout the ventricular zone and cortical plate in the majority of cells. (Figure 5.1d).

Analysis of axon and dendritic morphology

HSPGs have been implicated in the regulation of axon guidance (Walz et al, 1997; Irie et al, 2002). HSPGs have also been shown to influence thalamic neurite outgrowth suggesting that they may play a role in the formation of the thalamocortical pathway

(Kinnunen et al, 1999). E19 *Hs2st* mutant embryos were examined for evidence of any abnormalities of the thalamocortical axon tract (TCA) using DiI labelling to reveal the axons. As shown in Figure 5.2, TCA projections are present, and appear normal in homozygous mutant embryos. Due to the known involvement of heparan sulphate proteoglycans in the development of the thalamocortical pathway and in axonal pathfinding (Kinnunen et al, 1999; Walz et al, 1997) Golgi staining of the cerebral cortex was used to directly reveal axonal and dendritic morpology. Additionally, cytochrome oxidase staining of the barrel cortex was carried out (by F.Karlesson, according to the method of Seligman et al, 1968). This structure is formed by thalamic innervation of the sensory cortex which produces characteristic groups of cells which are alighned next to each other and relate to individual whiskers on the whisker pad of the mouse. As normal dendritic fields are necessary for the formation of this structure any abnormality in these dendrites would be expected produce an absence of barrels, or a blurring of the edges between individual barrels. Golgi staining and cytochrome oxidase was carried out on $Hs2st^{+/+}$ and $Hs2st^{+/-}$ animals at P10, though no abnormalities were observed. Since homozygous mutant animals die shortly after birth, heterozygous animals (which showed no abnormal phenotype) were used to look at postnatal ages (Figures 5.3 & 5.4).

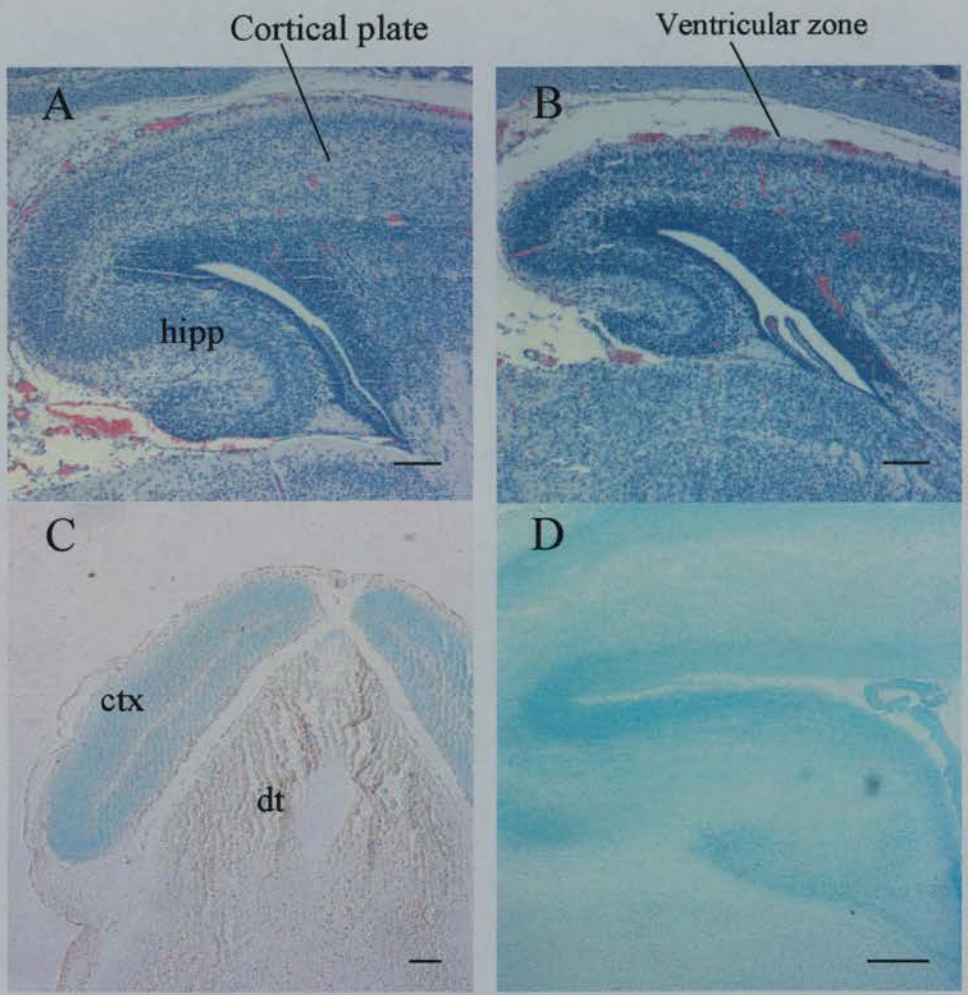


Figure 5.1 Histological analysis of the *Hs2st*$^{-/-}$ mutant and lacZ staining, showing areas of *Hs2st* expression in the embryonic forebrain. (A-B) Coronal sections of the cerebral cortex in E19 *Hs2st*$^{+/+}$ and *Hs2st*$^{-/-}$ mice respectively, counterstained to reveal cell density show a marked reduction in thickness of the cortex in the mutant. (C-D) LacZ staining in a coronal section of cerebral cortex from a *Hs2st*$^{+/-}$ embryo at E12 and horizontal section at E19.5 respectively. Staining indicates widespread expression of *Hs2st* at E19 and E12 in the cortex but not in the dorsal thalamus at E12. Abbreviations: Ctx, cerebral cortex; dt, dorsal thalamus; hipp, hippocampus. Scale bars, 100μm. (work carried out by Dr. V.Wilson)

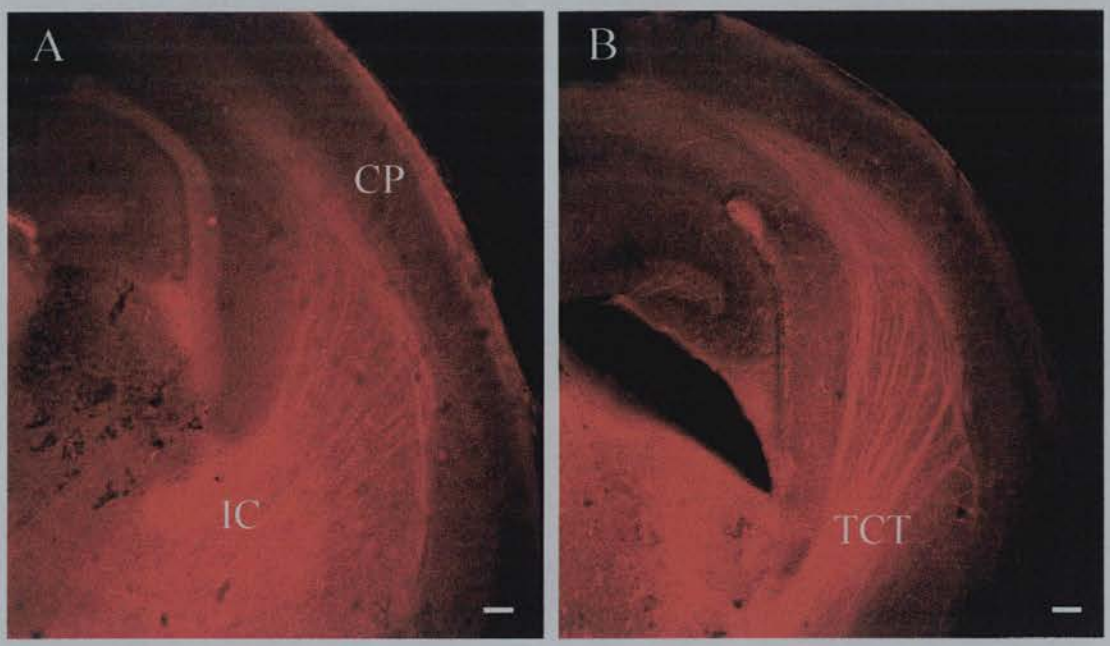


Figure 5.2 DiI labelling of the thalamocortical tract in sagittal sections at E19. (A) Section from an *Hs2st*$^{+/+}$ embryo showing the normal growth of the thalamocortical tract. (B) Section from a *Hs2st*$^{-/-}$ embryo showing the thalamocortical tract growing as normal and penetrating into the cortical plate. Abbreviations: CP, cerebral cortical plate; IC, internal capsule; TCT, thalamocortical tract. Scale bars, 100μm.

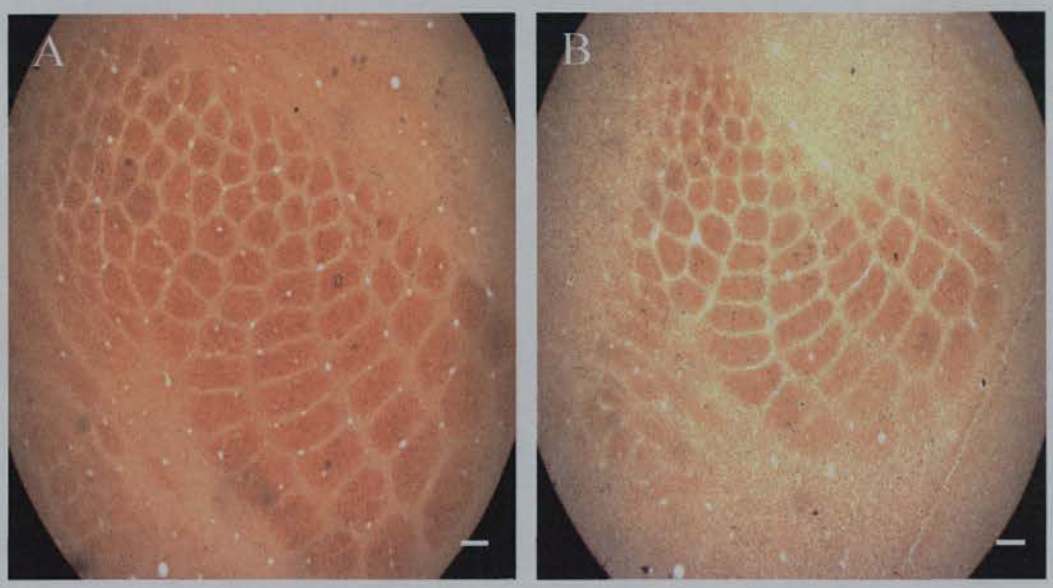


Figure 5.3 Cytochrome oxidase staining in flat mount preparations of the barrel cortex in P10 animals. (A) Section showing normal distribution and spacing of the barrel fields in the cortex in an *Hs2st*$^{+/+}$ animal. (B) Comparable section showing normal barrel fields in a *Hs2st*$^{+/-}$ animal. Scale bars, 100μm.

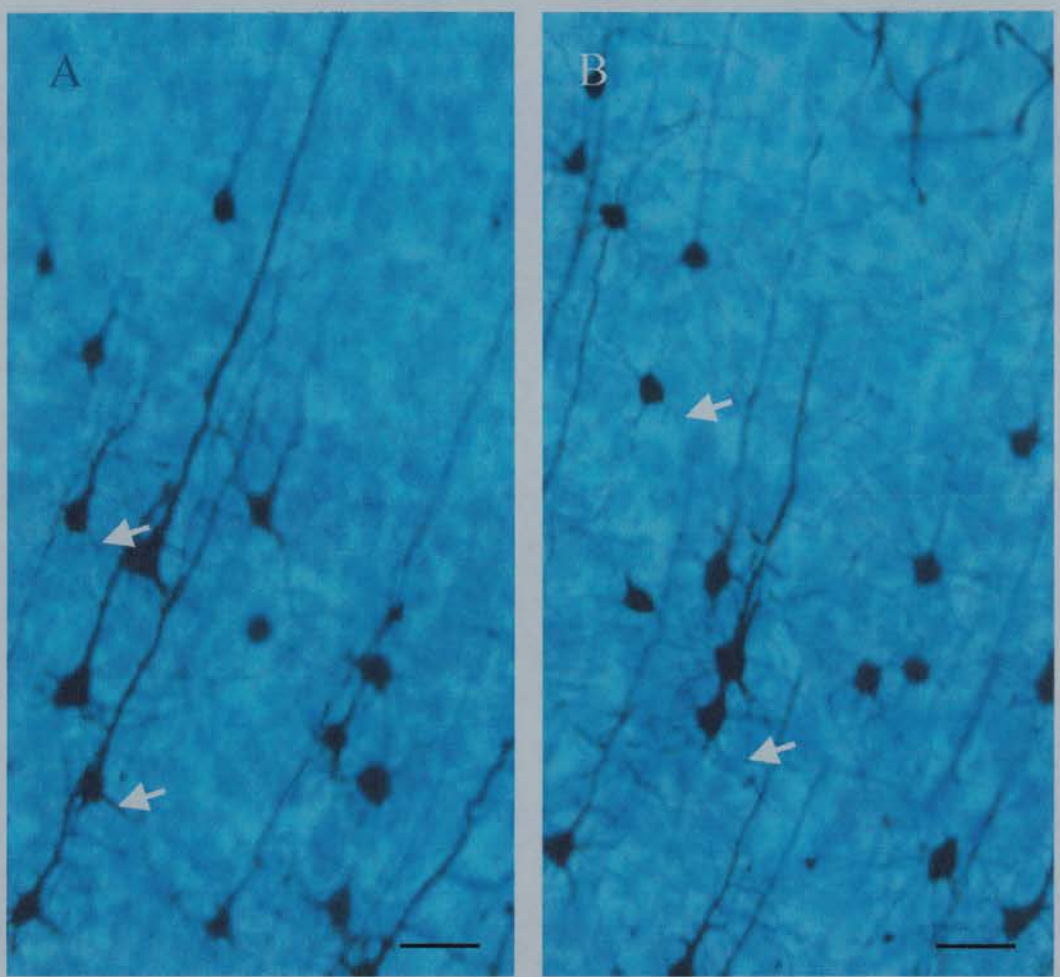


Figure 5.4 Golgi Cox staining of sagittal sections of the cerebral cortex in P10 animals. (A) Section of an *Hs2st*$^{+/+}$ animal showing pyramidal neurons with prominent axonal processes and dendritic trees (arrows). (B) Comparable section from an *Hs2st*$^{+/-}$ animal showing similar axonal and dendritic processes from pyramidal neurons, arrows. Scale bars, 100μm.

5.4.2 Cell proliferation in hs2st$^{-/-}$ cortex

To determine whether alterations in proliferation of neural precursor cells contribute to the reduced size of the cortex in *Hs2st* mutants, the labelling index (LI) was determined in regions of the embryonic cortex. Proliferation was examined in the dorso-medial wall of the telencephalon at E12.5. Three pairs of E12 *Hs2st*$^{+/+}$ and *Hs2st*$^{-/-}$ littermates were labelled with a single four hour pulse of BrdU, and sacrificed one hour after the last injection, such that it is highly unlikely that any cell would re-divide between the time of injection and sacrifice, thereby allowing classification of cells simply as BrdU positive or negative.

Camera lucida drawings were made of the dorso-ventral wall of every tenth section throughout the rostro-caudal axis of the telencephalon. In each section a reference point was chosen at the junction between the neuroepithelium of the neocortex and choroid plexus (similar to the method of analysis used in section 3.4.3) (Figure 3.8). This point is anatomically recognisable due to a marked thinning of the epithelium at this junction. On sections rostral and caudal to the choroid plexus, a thinning of the medial wall was still observable and in these cases an equivalent continuation point was chosen. From this reference point, to the dorsal-most aspect, the epithelium was divided into 100μm bins. BrdU labelled and non labelled cells were counted in each of these bins and these data used to generate labelling indices (LI) as described in section 3.4.3. The LI was plotted against position, either rostro-caudally or anterior-posteriorally (Figure 5.6).

Labelling indices were plotted to produce a profile of proliferative rates along the dorso-ventral and rostro-caudal axes of the medial wall of the telencephalon. The LI in *Hs2st*$^{-/-}$ mutant embryos is consistently lower than that in wild type embryos along the full extent of the anterior-posterior axis of the medial wall of the telencephalon (Figures 5.5 and 5.6). The LI in both *Hs2st*$^{-/-}$ and wild type embryos increases progressively from ventral to dorsal, consistent with the increasing thickness of the tissue along this axis resulting from increased precursor proliferation. Along the rostro-caudal axis of the medial telencephalic wall, the LI in *Hs2st*$^{-/-}$ mutant embryos is also consistently lower than that in wild types. In contrast to the medio-lateral axis, no obvious trends are seen in proliferative rates along the rostrocaudal axis, either in wild type or *Hs2st*$^{-/-}$ embryos.

Labelling indices in the lateral cortex from E12 *Hs2st*$^{-/-}$ and *Hs2st*$^{+/+}$ littermates were also measured. For each animal, three serial coronal sections from the mid-point along the rostro-caudal axis of the cortex were analysed (see Figure 3.8.b). From half way along the dorso-ventral axis of the lateral wall of the cortex in each section, an area 100μm wide, from the ventricular edge to the pial edge was designated for analysis. Numbers of BrdU labelled and unlabelled cells from the three serial sections were used to generate labelling indices for each animal. A significant reduction in the LI of *Hs2st*$^{-/-}$ embryos compared to wild type was observed (Figure 5.6c)(see also Figure 5.5). No difference in LI was seen in the dorsal thalamus which does not express *Hs2st* (Figure 5.6c).

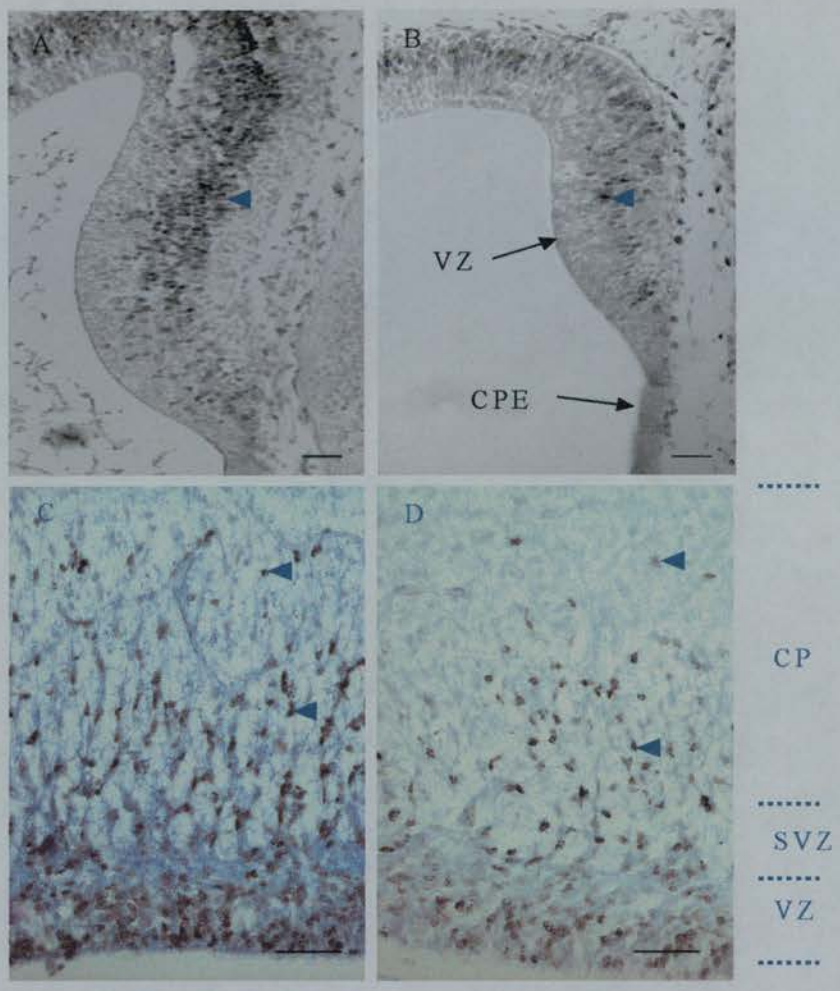


Figure 5.5 Coronal sections from E12 and E19 embryos which were injected with BrdU. (A,B) Sections from the medial wall of the telencephalon of an E12 *Hs2st*$^{+/+}$ embryo, and an homozygous mutant littermate, respectively. Darkly labelled cells indicate BrdU immunoreactivity. (C) Section from a E19 *Hs2st*$^{+/+}$ embryo injected with BrdU on E17 showing many cells labelled positive for BrdU (darkly coloured arrows). Density of labelled cells is seen to be highest in the ventricular zone. (D) A matched section from a E19 *Hs2st*$^{-/-}$ littermate embryo showing a marked reduction in the number of BrdU labelled cells present. (C,D counterstained with crystal violet). Distribution of labelled cells is mainly restricted to the ventricular and subventricular zones similar to wild type embryos. Abbreviations: CP, cortical plate; CPE, choroid plexus epithelium; SVZ, subventricular zone; VZ, ventricular zone. Scale bars, 50μm.

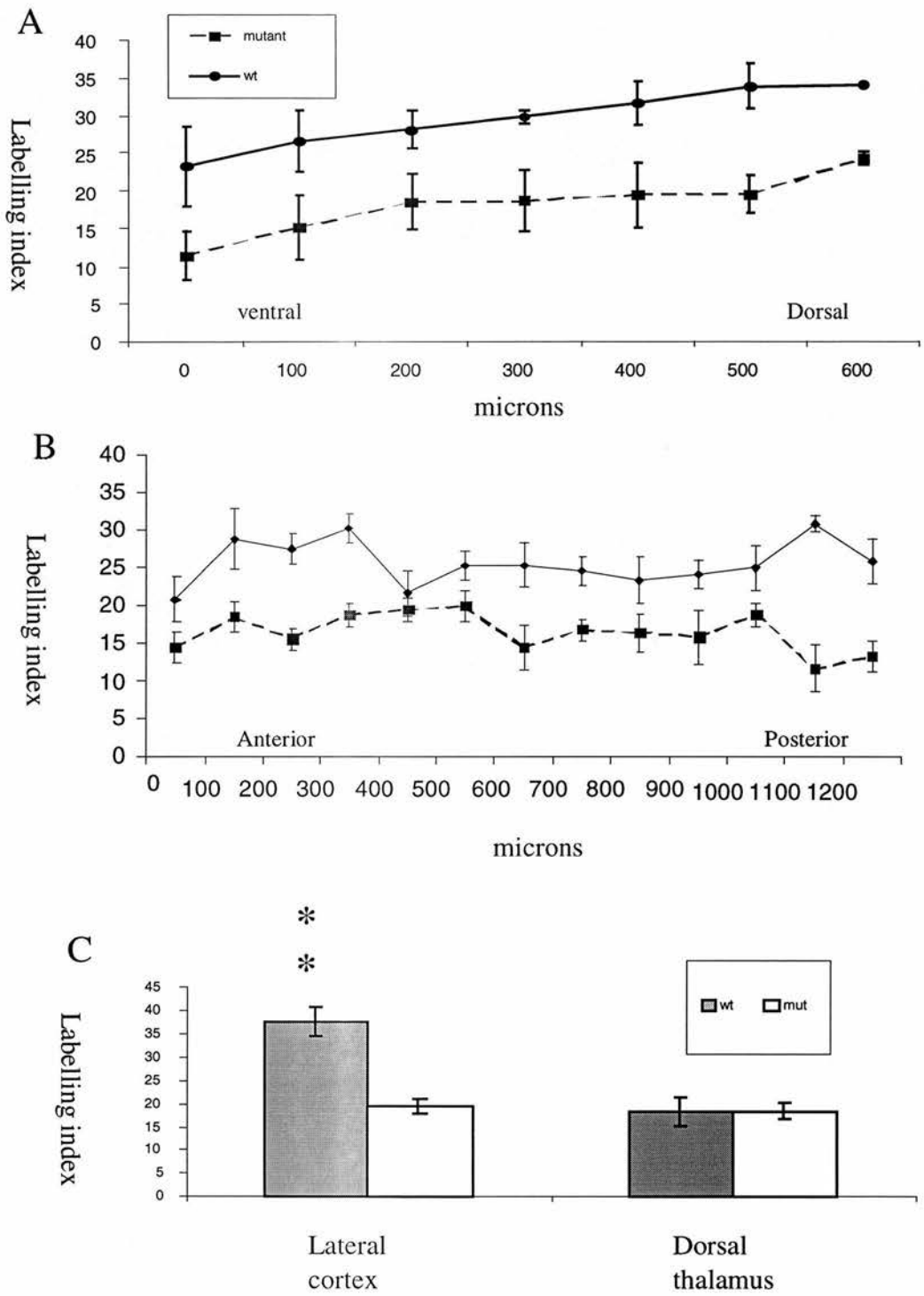

A
mutant
wt
Labelling index
40
35
30
25
20
15
10
5
0
ventral
Dorsal
0
100
200
300
400
500
600
microns
B
Labelling index
40
35
30
25
20
15
10
5
0
Anterior
Posterior
0
100
200
300
400
500
600
700
800
900
1000
1100
1200
microns
C
*
*
Labelling index
45
40
35
30
25
20
15
10
5
0
wt
mut
Lateral cortex
Dorsal thalamus

Figure 5.6 Effects of *Hs2st* on the proliferation of cortical precursor cells. Labelling indices for E12 $Hs2st^{+/+}$ and $Hs2st^{-/-}$ embryos, four hours after a single injection of BrdU, ($\pm$ s.e.m) plotted against distance along the dorso-ventral axis (A) and the anterio-posterior axis (B). Mutant data denoted by dashed lines and wild type by solid lines. (C) Bar graph showing the labelling indices for $Hs2st^{+/+}$ and $Hs2st^{-/-}$ embryos at E12 in the lateral cortex and dorsal thalamus. (**=$P<0.01$). Filled boxes denote data for $Hs2st^{+/+}$ embryos. Abbreviations: wt; wild type; mut, mutant.

5.4.3 Cell migration in hs2st$^{-/-}$ cortex

In order to determine the fates of cortical precursor cells we employed BrdU birthdating analysis. Embryos were pulse-labelled with BrdU at E13, E15 or E17 then sacrificed at E19 and the positions of BrdU labelled cells determined (Figure 5.7). BrdU labelled cells in these embryos were classified into two groups, heavily labelled cells (more than half the nucleus covered by reaction product) and lightly labelled cells (less than half the nucleus covered by reaction product). Previous work has shown that heavily labelled cells are those born on the day of injection (first generation), whereas the majority of lightly labelled cells are the product of subsequent progenitor cell divisions (second and third generation cells; Gillies and Price, 1993).

From sections, high power images of the lateral most point of the neocortex, from comparable levels of each animal were captured using a digital camera. The neocortical wall was divided into 10 equal bins from the ventricular to pial edge which were 100μm wide. The numbers of dark and lightly labelled cells were counted in each bin and averaged across mutant and wild type animals in each group (n=3). These figures were plotted against cortical depth (Figure 5.7). Means were compared statistically using Student's t-test.

The positions on E19 of cortical cells born on E13, E15 and E17 in wild type and *Hs2st*$^{-/-}$ mutant embryos are shown in Figure 5.7. In all 3 groups, E13, E15 and E17 born cells,

both heavily and lightly labelled wild type and *Hs2st*$^{-/-}$ cells were found in broadly similar positions in the wall of the telencephalon by E19.

E13 born cells, which had most time to migrate, showed a more skewed distribution than those born on E15. Heavily labelled cells were located mainly around the middle of the cortical plate whereas lightly labelled cells were mostly found in the superficial layers of the cortical plate. No obvious differences in position were seen between mutant and wild type animals (Fig 5.7A,B). The fates of heavily and lightly labelled cells born on E15 differed by E19. Heavily labelled cells were spread throughout the wall of the telencephalon, there being a higher density at the marginal zone. Many more lightly labelled cells were present at E15, also spread throughout the wall of the telencephalon, but distributed largely in the ventricular and marginal zone, and the superficial layers of the cortical plate (Figure 5.7c,d). Cortical cells born on E15 are fated to form a more superficial layer of the cortex than those born on E13 - consistent with this the densely labelled E15-born cells had, on average, migrated further than those born on E13. Both light and dense labelled E17 born cells were mainly restricted to the ventricular and intermediate zones, reflecting the much shorter time available for migration (Figure 5.7e,f). Taken together, these data indicate that migration of cortical precursor cells is unaffected in *Hs2st*$^{-/-}$ embryos.

Although the overall distributions of BrdU labelled cells was similar in *Hs2st*$^{-/-}$ and wild type embryos, some differences were observed. The highest density of both light and densely-labelled E17 born cells was found in the deepest layers, corresponding to the

ventricular and sub-ventricular zones. In both cases, the density of labelled cells in this layer was significantly lower in *Hs2st*$^{-/-}$ embryos than in wild type embryos (Figure 5.5c,e). This most likely indicates that proliferative rates remain lower in mutant embryos at later stages of neurogenesis. In the cortical plate and marginal zones where fewer labelled cells were found, no differences in density were seen (Figure 5.7e,f).

The densities of heavily labelled E15-born cells were similar in wild type and *Hs2st*$^{-/-}$ mutant embryos (Figure 5.7c). However, lightly-labelled *Hs2st*$^{-/-}$ cells were found at lower densities than corresponding wild type cells at all cortical depths (Figure 5.7d). This difference was most pronounced in the ventricular zone, again probably reflecting a lower proliferative rate in the mutants. Perhaps more surprisingly, the densities of both heavily and lightly labelled cells born on E13 were similar in wild type and *Hs2st*$^{-/-}$ mutant embryos at all cortical depths. This might suggest the presence of some sort of compensatory mechanism. These data reinforce the hypothesis of a proliferative defect, and possibly indicate an attempt to compensate for this by the production of more neuronal precursor cells.

Figure 5.7

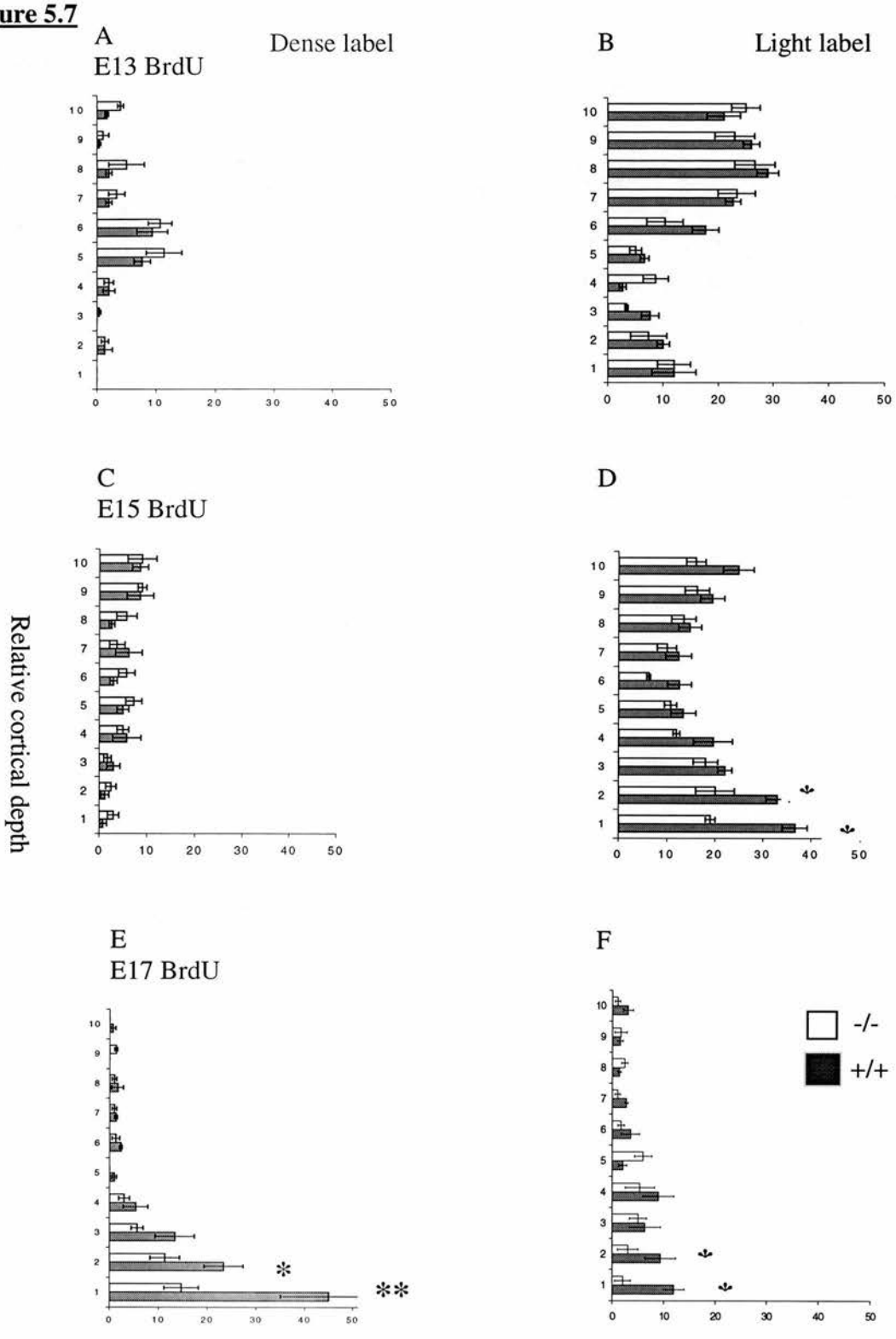

A
E13 BrdU
Dense label
B
Light label
C
E15 BrdU
D
E
E17 BrdU
F
-/-
+/+
*
**
Relative cortical depth
Number of BrdU labelled cells

Figure 5.7 Effects of *Hs2st* on the migration of cortical precursor cells. Histogram plots of the average densities (± s.e.m) of light and darkly labelled cells in E19 embryos against depth of the lateral cortex from ventricular zone to pial edge (50X100μm bins). Mice were injected with BrdU on E13 (A,B), E15 (C,D) or E17 (E,F). (*P<0.1 **=P<0.01). Filled bars denote data for $Hs2st^{+/+}$ while open bars denote data for $Hs2st^{-/-}$. N=3-6.

5.5 Discussion

Histological examination of Hs2st$^{+/+}$ and Hs2st$^{-/-}$ mice, shows that the size of the cortex is dramatically reduced in Hs2st$^{-/-}$ animals (see Figure 5.1a,b). As described in Chapter 1, cells born in the ventricular zone of the cortex migrate to form the cortical plate, expanding the cortex in the process. This difference in size may be due to differences in migration, proliferation or cell death.

No apparent migratory differences were found between *Hs2st*$^{-/-}$ and *Hs2st*$^{+/+}$ embryos though these data were compatible with higher proliferation in wild type embryos injected at E15 and E17 (statistically significant differences found in the numbers of dark and lightly labelled cells in the ventricular zone). Data obtained for embryos injected at E13 however showed no significant difference in LI between *Hs2st*$^{-/-}$ and *Hs2st*$^{+/+}$ embryos. In this set of embryos, BrdU was present for the longest time before sacrifice and therefore there was more potential for redivision of cells which had taken up this marker. After many divisions of a cell containing BrdU, a dilution of BrdU may be expected to occur to a point when detection is no longer possible. As cortical thickness in *Hs2st*$^{-/-}$ embryos is less than in wild type littermates at E19, a dilution of BrdU due to extensive proliferation seems unlikely since in this case cortical thickness in *Hs2st*$^{-/-}$ embryos would be expected to be at least equivalent to that of the wild type. It is possible that the results at E13 represent a compensation by the brain for the reduction in proliferation earlier on during development, by mechanisms other than Hs2st-aided signalling, perhaps by a shift towards neurogenic cell division by stem cells in the ventricular zone to produce increased numbers of neuronal precursors. The

interpretation of such BrdU data on migration is problematic however, as a large proportion of cells migrate into the cortex from the ganglionic eminence during this time and subsequently differentiate into interneurons (E13 to early post natal ages)(Anderson et a., 2002). In the absence of specific markers these cells may be confused with radially migrating cells from the cortical ventricular zone which subsequently differentiate into cortical projection neurons. Hence, any effect on cell migration observed in such a BrdU labelling study can not distinguish between radial and tangentially migrating cells (ie. The site of the effect, cortical or sub-cortical). Additionally, an effect on layer specific migration as measured here may be accounted for by a cell proliferation defect either in the cortex or sub-cortex.

Other aspects of cortical development were found to be unaffected in *Hs2st*$^{-/-}$ embryos. DiI labelling of the thalamocortical tract in *Hs2st*$^{-/-}$ and *Hs2st*$^{+/+}$ indicates that this structure develops normally in the mutant. As homozygous *Hs2st*$^{-/-}$ mutants die around the time of birth (for reasons thought to be unconnected to this neural phenotype) analysis of proliferation or axonal and dendritic formation at later stages is not possible. However, aspects of connectivity were examined in heterozygous mutants and wild type pups at P10 using Golgi Cox and cytochrome oxidase staining (work carried out in collaboration with Frederick Karlsson). In heterozygous animals, Golgi staining in the lateral cortex revealed no difference in cell type, axonal or dendritic processes. Cytochrome oxidase staining of the barrel fields also revealed no differences which might be expected if dendritic formation is affected. Recently, it has been shown that distinct saccharide structures and O-sulphation of HSPGs is necessary for the normal

guidance of axons from retinal ganglion cells in Xenopus. In addition, it has been sugested that the expression of *Hs2st* and *Hs6st* may be important in generating distinct heparan sulphate structures in the dorsal diencephalon which may be critical for steering axon growth into the tectum (Holt et al, 2002).

It is likely that in *Hs2st* heterozygous mice, enough enzyme is still produced in order for normal development to proceed. *In vivo,* it would be possible to generate an inducible *Hs2st*$^{-/-}$ in which tissue specific expression of *Hs2st* in the cerebral cortex could be switched off at critical periods of postnatal development to investigate the effects on axonal formation. Additionally, a *Hs2st*$^{-/-}$ *Hs6st*$^{-/-}$ double knockout may uncover where a combination of 2-0 and 6-0 sulphation is necessary for development, where either 2-0 or 6-0 sulphation alone is not.

Circumstantially, widespread *Hs2st* expression occurs over the period of cell generation when the process of connection formation is not yet at its height. Although heparan sulphate is involved in many growth factor signalling pathways, as described in the introduction, the phenotype observed in the *Hs2st*$^{-/-}$ embryos is most likely due to a disruption in FGF signalling. Firstly, the expression pattern and timing of expression of other growth factors implicated in heparan sulphate action in many cases does not match that of *Hs2st*. For example, multiple members of the *Wnt* gene family are expressed in the cortex and their role in proliferation is well documented (see chapter 1). However, these genes are expressed in restricted domains which do not encompass the entire cortex, and while it is possible that their effects are exerted at a distance which is

feasible at E12, tissue thickness at later stages would make this more difficult. Secondly, the timing and widespread expression of FGFs over the period of cortical neurogenesis, together with the potent proliferative effects of FGF, point to the involvement of this signalling pathway.

Mice deficient in FGF2 have been described (Dono et al, 1998; Ortega et al, 1998), and the effect of loss of FGF2 upon cortical development has been examined in detail (Vaccarino et al, 1999; Raballo et al, 2000). In the absence of FGF2, cortical precursor cells in the ventricular zone of the dorsal cortex in E12 mice are grately reduced, both in number and in proliferative rate.

The biochemical phenotype of primary embryonic fibroblasts derived from these Hs2st mutant mice has been investigated (Merry et al 2001). HSPGs isolated from such cells completely lack uronate 2-O-sulphates, demonstrating that the gene-trap induced mutation is a genuine null mutation. They also demonstrated much reduced binding to FGF1 and FGF2, but not HGF in a filter-binding assay. Surprisingly, however, no reduction in FGF2 responsiveness could be detected in the mutant cells suggesting that in this cell type, potentiation of FGF1 and FGF2 signalling by heparan sulphate does not necessarily require high affinity binding.

5.6 Conclusions

The results suggest that the reduction in cortical size in mice lacking Hs2st most likely arises as a consequence of significantly reduced rates of proliferation of cortical precursor cells, though the effect of cell death was not ruled out.

These data provide clear evidence that 2-0 sulphation of heparan sulphate proteoglycans is necessary for normal cortical cell generation during early embryogenesis. Cortical cell migration appears to be normal in the absence of *Hs2st.*

The effects of *Hs2st* in the cerebral cortex appear consistent with defects in FGF2 signalling which are known to affect proliferation of cortical precursor cells. Other processes such as axon pathfinding and cell inter-connectivity which may be affected by heparinase activity, are normal in $Hs2st^{-/-}$ mice, as indicated by the DiI, Golgi and cytochrome oxidase staining. Selective effects are likely to reflect the activities of specific HS sequences, and an analysis of the temporal and spatial expression of these sequences will be necessary in identifying such effects in other developmental processes.

CHAPTER 6. Final discussion

In this thesis the role of a number of different components of the Wnt signalling pathway during forebrain development and adulthood were investigated. Co-expression of *Wnt10B* and *Frizzled1* in brain and limb development were also examined, though this data was incomplete and therefore not included. Previous work has focused largely on the identification of *Wnt* gene family members and related signalling molecules such as Frizzled receptors. Ninteen members of the *Wnt* gene family have been identified so far, and with the completion of the Human genome project (19 Human Wnt family members identified) it is likely that this complement will not increase dramatically.

The function of many of these genes still remains to be elucidated and provides the current challenge for future research. The dynamic nature of expression of these genes is clear however, equally dynamic functional relationships during development have only recently been demonstrated (Niehrs, 2001) and indicate the complexity of the task. This work has shown that in the case of one Frizzled receptor, the presence of different Wnt ligands is necessary in directing which of the three signalling pathways is stimulated. The functional relationships of similar interactions in specific tissues, sets and subsets of cells during development and adulthood will need to be determined if we are to gain a full understanding of development, and mature processes in adulthood.

Work presented in this thesis has attempted to determin the functional role of a number of such genes. In the time alowed only some aspects of function such as cell

proliferation have been examined, however this should provide a basis for continuing experiments. In Chapter 3, I investigated the role of the *Wnt8B* during embryogenesis from E7.5 to E14.5 using *in situ* hybridisation and BrdU labelling studies in $Wnt8B^{+/+}$ and $Wnt8B^{-/-}$ mice. The expression pattern of *Wnt8B* in the mouse had not been described in the literature and its function was unknown. A restricted domain of strongly expressing cells in the medial wall of the telencephalon was found, though subsequently no effect on cell proliferation was observed in this region at E12.5 –E14.5. Multiple members of the *Wnt* gene family are expressed in the medial wall of the telencephalon (Grove et al, 1998), some of which have been found to be important in the patterning of the hippocampus which develop from this area (Lee et al, 2000; Galceran et al, 2000). As the expression of these genes occurs in overlapping patterns, and is highly conserved, it is likely that they may co-operate with one another to exert their effects on the development of this region. To evaluate this, *in vitro* and *in vivo* analysis of the effects of removing combinations of these genes will be necessary, in addition to an understanding of the timing of expression of Frizzled receptors and inhibitory molecules which are present during embryogenesis in the medial telencephalic wall.

In Chapter 4, I investigated the role of the *Wnt8B* in adulthood using *in situ* hybridisation and BrdU labelling studies in $Wnt8B^{+/+}$ and $Wnt8B^{-/-}$ mice. The expression pattern of *Wnt8B* in the mouse had not been described. Surprisingly, I found multiple sites of restricted expression which included the subgranular layer of the hippocampus, an area of continued neurogenesis during adulthood (Altman, 1965; Gould et al, 1999). It is possible that the production of these cells is necessary in normal memory function,

however the factors which influence this production are not known. In light of the proliferative effects of many members of the *Wnt* gene family (Nusse & Varmus, 1982), I carried out a careful study of BrdU labelling in this area. The results were consistent with an increase in the survival of newly born subgranular layer cells after four days, though difficulties were encountered in generating sufficient numbers of mice and in designing the BrdU protocol, as well as controlling for many other factors. The future direction of experiments may focus on other possible effects of Wnt8B in the adult brain such as synaptic plasticity and cell adhesion, suggested to be altered in a number of psychiatric disorders, due to abnormal Wnt signalling (Hall et al, 2000).

In Chapter 5, the effects of a lack of heparan sulphate 2-0 sulphotransferase (Hs2st) were investigated using a Hs2st knockout mouse (Bullock et al, 1998). Hs2st is one of three enzymes which sulphates glycosaminoglycan chains, which have recently been found to be involved in a number of important signalling pathways (including Wnt signalling) (Rapraeger et al, 1991;Itoh & Sokol, 1994; Bellaiche et al, 1998). The specific interactions of these molecules is determined, in part, by the sulphation pattern of the glycosaminoglycan chain (Salmivirta et al, 1996) and therefore, analysing the effects of removing one of these enzymes is important in understanding the effects of specific chain modification on development. Histological analysis and a subsequent BrdU study in $Hs2st^{+/+}$ and $Hs2st^{-/-}$ mice found that cell proliferation in the cerebral cortex was dramatically reduced in $Hs2st^{-/-}$ mice. The direction of future work will be to further examine the effects of different sulphation patterns of heparan sulphate proteoglycans,

for example, to generate Hs2st$^{-/-}$ Hs6st$^{-/-}$ double knockout mice, and to more specifically determine which signalling pathways these molecules interact with.

Additional to the presented work, I investigated the expression of *Wnt10B* and the Frizzled-1 receptor during early development of the mouse. The expression pattern of *Wnt10B* had been published, however, preliminary work suggested that these two genes were co-expressed at least at one site. This work was extended using *in situ* hybridisation and further sites of co-expression in the limb and brain were found. Limited time precluded a more thorough study though these preliminary results indicated that *Wnt10B* and *Frizzled-1* may form a ligand-receptor relationship.

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Appendix 1

DNA extraction

Tail tip lysis buffer (TTLB)	100mM Tris pH8.0 200mM NaCl 0.5mM EDTA 0.2% SDS
Tris-EDTA	0.1M Tris pH7.4 1mM EDTA
Phosphate buffered saline (PBS)	PBS tablets (Oxoid:BR14) 1 tablet dissolved in 100mls water

Southern blot solutions

Denaturing solution	0.5M NaOH 1.5M NaCl
Neutralisation buffer (pH to neutral)	0.5M Tris pH7.0 3M NaCl
20XSSC	3M NaCl 0.3M Sodium Citrate
Hybridisation solution	1mM EDTA O.5M NaHPO4 7% SDS 1% BSA
Church wash solution	40mM NaHPO4 1mM EDTA 1% SDS

In situ hybridisation solutions

20XSSPE (pH7.4)	3M NaCl 2M NaH2PO4 0.02M EDTA
20XSSC (pH7.0)	3M NaCl 3M Sodium Citrate
10XPBS (pH7.4)	1.37M NaCl 27.6mM KCL 81mM Na_2HP0_4 14.7mM KH_2P0_4
PTW / PBST	0.1% Tween 20 in 1XPBS

20X P-buffer (pH7.5)	1M Tris/HCL 0.1M EDTA

BrdU immunocytochemistry solutions

Stock Tris	100mM Tris. PH7.6
TBS	200mM NaCl 10mM stock Tris
TBST	TBS 0.1% Tween 20

Golgi solutions

Golgi solution	100mM K2Cr207 100mM HgCl2 10mM K2CrO4
Golgi Buffer	10mM NaAc pH with Acetic acid to 4.1

Appendix 2

Chapter 4. Experiment 4. Numbers of BrdU labelled cells in coronal sections along the rostro-caudal axis of the cortex, from male and female *Wnt8B*$^{+/+}$ and *Wnt8B*$^{-/-}$ mice, in the subgranular cell layer, granular cell layer, Hilus, CA fields and the Z area.

Subgranular Zone. Male

	0		24hrs			48hrs	72hrs	
Wnt8B$^{+/+}$								
0 microns	5	1	10	0		5	0	1
100	0	2	6	1		2	3	3
200	1	1	4	3	13	9	18	3
300	7	9	9	5	10	10	15	3
400	2	6	10	1	7	9	11	2
500	0	13	8	2	7	12	7	4
600	18	8	14	4	9	11	17	3
700	6	5	4	3	4	10	5	3
800	9	14	6	4	12	13	9	3
900	3	8	5	4	8	6	14	1
1000	1	3	11	2	4	6	14	1
1100	5	5	9	4	12	10	5	1
1200	4	8	1	0		7	7	6
1300	3	12	6	1		4	3	5
1400	6	19	7	3		9	3	

Wnt8B$^{-/-}$	0		24hrs		48hrs		72hrs	
0	1	9	12	6	1	2	0	2
100	1	4	6	4	2	2	11	10
200	6	3	5	4	6	3	12	5
300	6	12	5	3	2	10	16	6
400	2	0	4	1	9	9	11	1
500	10	7	6	6	9	9	8	2
600	8	7	3	5	9	5	12	3
700	15	2	4	6	4	1	4	3
800	6	3	12	6	5	5	6	7
900	7	3		3	2	7	14	2
1000	10	5		8	1	3	5	0
1100	2	1			3	7	5	3
1200	4	4			8	5	13	3
1300	4	3			8	5	10	9
1400	11	5			1	9	9	3

Subgranular Zone, Female

Wnt8B$^{+/+}$	0	24hrs		48hrs	72hrs
0	1	7	10		3

100	1	8	13		0
200	0	6	10		1
300	2	6	11		13
400	4	5	11		10
500	4	12	19		3
600	4	8	9		7
700	6	18	11		7
800	10	15	11		3
900	3	17	7		11
1000	4	3	8		2
1100	7	10	5		9
1200	7	6	3		1
1300		7	13		
1400		5			

Wnt8B$^{-/-}$	0	24hrs		48hrs			72hrs	
0	2	2	0	5	0	10	0	7
100	5	1	7	7	3	18	7	0
200	8	7	4	5	4	5	9	4
300	7	1	3	2	9	2	6	13
400	3	14	14	4	13	8	14	4
500	5		12	8	8	8	9	4
600	6	8	9	10	8	6	7	2
700	6	8	11	12	4	16	4	19
800	8	12	10	7	10	12	7	10
900	8	3		2	3	11	7	7
1000	4	6		5	3	4	6	4
1100	4	4		5	5	11	5	4
1200	6	4		5	8	6	11	9
1300	8	8		6	5	4	8	4
1400	6	4		7			8	4

Granular Zone, male

Wnt8B$^{+/+}$	0		24hrs			48hrs	72hrs	
0	0	3	0	0		1	1	0
100	3	0	3	0		2	0	0
200	0	0	0	0	2	0	1	1
300	0	4	1	0	1	0	1	0
400	1	4	2	1	3	1	3	0
500	0	0	1	0	0	2	2	0
600	1	0	0	0	3	7	1	1
700	3	3	1	0	1	1	1	1
800	0	1	0	0	1	1	2	0
900	0	3	0	0	4	5	0	2
1000	1	0	0	0	4	4	3	0
1100	3	1	0	0	2	2	2	1
1200	1	4	4	0		2	0	3

1300	0	6	1	0		3	3	1
1400	0	1	1	0		1	1	

Wnt8B$^{-/-}$	0		24hrs		48hrs		72hrs	
0	0	0	2	0	0	0	1	2
100	3	2	4	0	2	0	2	0
200	2	0	0	0	0	1	0	1
300	1	1	3	0	0	3	0	2
400	0	0	1	0	0	1	1	1
500	1	3	0	2	0	1	1	0
600	2	1	1	1	1	1	3	3
700	1	0	1	0	3	2	0	2
800	2	1	0	3	0	0	2	0
900	1	1		1	1	0	1	2
1000	1	0		1	0	1	0	0
1100	1	0			0	1	0	0
1200	3	2			0	4	0	4
1300	2	0			0	2	2	3
1400	1	2			0	0	1	1

Granular Zone, female

Wnt8B$^{+/+}$	0	24hrs		48hrs	72hrs
0	0	0	0		0
100	0	5	0		1
200	0	0	0		2
300	0	0	2		1
400	0	3	0		3
500	0	0	1		0
600	0	0	1		0
700	0	4	3		1
800	2	3	1		0
900	1	0	2		0
1000	1	1	2		0
1100	1	1	3		0
1200	3	1	2		0
1300	0	0	4		
1400		2			

Wnt8B$^{-/-}$	0	24hrs		48hrs			72hrs	
0	0	0	0	0	0	0	0	0
100	1	0	0	0	0	0	1	1
200	0	1	0	2	0	1	1	1
300	2	0	0	0	0	2	1	0
400	0	2	0	2	0	3	5	1
500	2		4	1	0	1	1	2
600	0	0	2	1	1	0	1	1
700	0	1	1	1	0	1	0	0
800	0	1	1	3	1	0	0	2

900	1	0		0	0	0	2	1
1000	2	3		0	1	0	1	5
1100	2	0		1	0	0	0	0
1200	0	0		0	1	0	2	4
1300	0	0		1	0	0	0	1
1400	3	0		0			1	0

H AREA, male

Wnt8B$^{+/+}$			24hrs			48hrs	72hrs	
0	0	0	1	0		1	0	0
100	0	0	0	0		1	0	0
200	0	0	0	0	0	0	0	0
300	1	2	0	0	0	0	1	0
400	0	0	0	0	0	0	1	1
500	0	0	0	0	0	0	1	0
600	0	1	0	0	2	0	3	0
700	0	1	0	0	0	1	0	0
800	0	2	0	0	0	0	3	0
900	0	0	0	0	0	0	1	0
1000	0	0	1	0	1	0	0	0
1100	0	0	0	0	0	0	0	0
1200	0	0	0	0		0	0	0
1300	0	0	1	0		0	0	1
1400	2	0	0	0		1	2	

Wnt8B$^{-/-}$	0		24hrs		48hrs		72hrs	
0	0	0	0	0	0	0	0	1
100	0	0	3	0	0	0	0	0
200	0	0	0	0	0	0	0	0
300	0	1	0	0	1	0	0	0
400	0	0	0	0	0	0	0	0
500	1	0	0	0	0	0	0	0
600	0	0	0	0	0	1	0	0
700	0	0	0	0	0	0	2	1
800	0	0	0	0	0	0	0	0
900	0	0		0	0	0	2	0
1000	0	0		0	0	1	2	1
1100	3	0		0	0	1	0	0
1200	0	0			0	1	1	0
1300	0	0			0	0	0	0
1400	0	0			0	0	4	0

H AREA, female

Wnt8B$^{+/+}$	0	24hrs		48hrs	72hrs
0	0	0	0		0

100	0	0	0		0
200	0	0	0		0
300	0	0	1		0
400	0	0	0		0
500	2	0	0		0
600	0	1	1		0
700	0	0	0		0
800	0	0	0		0
900	0	0	1		0
1000	0	0	0		0
1100	0	0	0		0
1200	0	0	0		0
1300	0	0	2		0
1400		0			

Wnt8B$^{-/-}$	0	24hrs		48hrs			72hrs	
0	0	0	0	1	1	0	1	0
100	0	0	0	0	3	0	0	0
200	0	0	0	0	0	0	0	1
300	0	0	0	0	0	0	1	0
400	2	0	0	0	0	0	1	0
500	0		0	1	2	0	0	1
600	0	0	0	0	0	0	0	0
700	0	0	0	0	1	0	0	1
800	0	0	0	0	0	0	0	0
900	0	1		0	0	0	0	0
1000	0	0		0	0	0	0	0
1100	0	0		1	0	1	0	1
1200	0	0		0	0	0	0	0
1300	0	0		0	0	0	0	0
1400	0	0		0			0	0

CA
Fields, male

Wnt8B$^{+/+}$	0		24hrs			48hrs	72hrs	
0	0	0	0	0		0	1	1
100	0	1	0	0		0	0	0
200	1	0	0	0	0	0	0	0
300	0	0	0	0	0	1	0	0
400	1	1	3	0	0	2	4	0
500	2	1	2	0	0	1	0	0
600	2	0	3	0	0	3	0	1
700	4	0	0	0	0	0	2	0
800	2	0	2	0	0	0	0	0
900	1	0	0	0	0	3	0	1
1000	0	0	0	0	0	1	0	0
1100	1	1	1	0		0	1	0

1200	1	0	0	0		0	0	2
1300	2	0	3	0		0	0	0
1400	2	0	0	1		0	0	

Wnt8B$^{-/-}$	0		24hrs		48hrs		72hrs	
0	1	0	1	0	2	0	2	1
100	0	0	1	0	0	3	0	0
200	2	0	1	0	2	1	0	0
300	0	0	0	0	0	0	0	0
400	0	0	2	0	2	0	0	1
500	0	0	0	0	5	0	1	0
600	0	0	0	0	0	1	0	1
700	0	0	0	0	0	0	1	0
800	0	2	0	0	0	0	1	2
900	3	0		0	1	1	1	1
1000	0	0		0	0	1	0	0
1100	2	1		0	0	0	1	0
1200	0	0			4	2	2	0
1300	0	0			4	2	1	0
1400	0	0			0	3	0	0

CA Fields, female

Wnt8B$^{+/+}$	0	24hrs		48hrs	72hrs
0	0	0	1		0
100	0	0	0		0
200	0	0	0		0
300	0	0	0		0
400	0	0	2		0
500	0	1	5		0
600	0	1	2		0
700	0	0	0		0
800	1	2	4		0
900	0	0	0		0
1000	1	1	0		0
1100	0	0	0		0
1200	0	0	1		0
1300		0	1		
1400		0			

Wnt8B$^{-/-}$	0	24hrs		48hrs			72hrs	
0	1	2	0	0	0	0	0	0
100	1	0	0	0	0	0	0	1
200	0	0	0	0	0	0	0	1
300	1	0	2	0	0	2	0	0
400	1	2	2	0	0	0	3	2
500	3		0	0	2	0	0	0
600	1	0	0	0	1	0	5	0

700	2	2	0	0	0	2	0	1
800	2	0	0	1	0	0	2	0
900	3	0	0	0	0	0	0	0
1000	4	1		0	0	0	0	0
1100	2	0		1	1	0	0	0
1200	3	0		3	1	0	0	2
1300	4	0		1	0	0	0	0
1400	3	0		1			0	0

Z Area, male

Wnt8B$^{+/+}$	0		24hrs			48hrs	72hrs	
0	0	2	1	0		3	0	0
100	0	1	3	0		4	1	0
200	1	0	2	0	0	1	1	0
300	0	2	4	0	5	0	6	1
400	1	2	5	0	3	0	5	2
500	2	3	7	0	1	1	2	1
600	2	5	2	0	2	3	1	4
700	4	7	4	0	3	5	4	1
800	2	3	3	0	3	3	2	0
900	1	4	1	0	0	0	2	1
1000	0	7	0	0	0	3	2	0
1100	1	6	3	0	1	6	7	2
1200	1	2	2	0		1	3	0
1300	2	6	5	0		4	4	0
1400	2	0	4	0		1	3	

Wnt8B$^{-/-}$	0		24hrs		48hrs		72hrs	
0	1	1	3	0	2	0	1	4
100	1	2	2	0	4	1	1	3
200	0	0	1	0	0	3	3	1
300	2	2	3	0	6	3	3	1
400	4	2	5	1	1	0	8	1
500	4	0	2	0	4	4	5	1
600	5	1	2	0	1	5	4	1
700	2	2	2	0	2	2	6	4
800	4	0	1	0	6	2	9	1
900	2	2		0	6	0	4	0
1000	2	0		0	4	0	3	3
1100	3	3			4	5	6	1
1200	1	1			3	1	6	7
1300	4	0			0	1	14	3
1400	3	2			2	3	2	4

Z Area, female

Wnt8B$^{+/+}$	0	24hrs		48hrs	72hrs
0	0	0	2		0
100	0	0	0		0
200	1	2	0		1
300	0	1	3		0
400	1	1	4		0
500	0	8	11		3
600	6	7	4		1
700	1	2	8		2
800	1	0	6		0
900	2	6	10		3
1000	3	1	6		4
1100	0	2	7		0
1200	2	0	3		2
1300		0	5		
1400		3			

Wnt8B$^{-/-}$ 0	24hrs		48hrs			72hrs		
0	1	0	1	0	0	1	0	2
100	1	0	5	2	0	3	2	0
200	0	0	2	4	1	0	2	5
300	1	3	0	5	0	0	9	1
400	1	2	1	2	2	2	6	4
500	3		6	3	2	2	0	3
600	1	1	1	0	1	2	1	5
700	2	2	3	5	4	7	2	1
800	2	1	9	3	3	1	8	4
900	3	3		0	2	0	1	7
1000	4	4		7	2	0	2	3
1100	2	0		2	0	3	2	3
1200	3	2		2	1	0	3	3
1300	4	1		3	3	3	1	1
1400	3	2		6			0	3